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Im Auftrag

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Piroplasmid vaccine

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Piroplasmid vaccine

The invention relates to a Piroplasmid protein or an immunogenic fragment of said protein, to a nucleic acid encoding said Piroplasmid protein or said immunogenic fragment, to cDNA fragments, recombinant DNA molecules and live recombinant carriers comprising said nucleic acid, to host cells comprising said cDNA fragments, recombinant DNA molecules and live recombinant carriers, to vaccines comprising a Piroplasmid protein or an immunogenic fragment of said protein, to methods for the preparation of such vaccines, to the use of such proteins or fragments, and to diagnostic tests.

Babesiosis is a disease, which has a geographically focal occurrence. The reason for this is that the pathogen is transmitted by ticks that feed on a certain reservoir of parasites present in a vertebrate population. Only where ticks are present, Babesiosis can occur. On balance, particularly in indigenous animals, the parasite coexists with the host without causing significant disease. In many cases Babesiosis becomes a problem because of man's activities through inbreeding of genetic traits and/or transporting animals to unfamiliar environments where Babesiosis is endemic (Callow, L.L. and Dalgliesh, R.J., 1982, in: "Immunology of Parasitic Infections", Cohen, S. and Warren, K.S. eds., p. 475-526, Blackwell Scientific).

Babesiosis also holds a threat as zoonotic agent for humans, not only to immunocompromised humans (Gray *et al.*, 2002, Int. J. Med. Microbiol., vol. 291, p. 108-11).

Signs of disease in naturally acquired Babesiosis usually begin 7-21 days after infection. These symptoms include: fever, anorexia, depression, anaemia, haemoglobinuria and rapidly developing weakness. Increased lacrimation, salivation and muscle tremor commonly occur. Nervous signs may develop in terminal infections, and death may occur when the disease is left untreated. Coagulation disturbances lead to increased erythrocyte-stickiness. As a result the blood passage through the microvasculature is hampered, resulting in congestion of internal organs and decreased packed cell volumes (PCV). Also rupture of infected erythrocytes causes loss of large numbers of erythrocytes. These effects impair the oxygen supply to several tissues and subsequently lead to tissue damage as a result of anoxia.

Species from the Babesiidae have now been detected to infect most mammalian species of veterinary importance (Kuttler, K.L., in M. Ristic ed.: "Babesiosis of domestic

animals and man". CRC Press, Inc., Boca Raton, FL, 1988): Cow (*B. divergens, B. bovis, B. bigemina*), Swine (*B. trautmanni, B. perroncitoi)*, Sheep (*B. ovis, B. motasi*), Horse (*B. equi, B. caballi*), Dog (*B. canis, B. rossi, B. vogeli*), and Cat (*B. felis, B. cati*). In all these species death or more or less severe economical losses (reduction in quality or quantity of meat, milk, wool, or offspring), or severe reduction in well-being are caused either as a result of the Babesia infection directly, or through facilitation of secondary infections.

Closely related to Babesia are Theileria parasites. These also belong to the taxonomic group of the Piroplasmida, and show many biological and epidemiological relationships to Babesia. Well known Theileria species of veterinary importance are *T. parva*, *T. annulata*, and *T. sergenti*.

Medications exist to cure an established Babesia or Theileria infection, for instance dogs, horses and cows can be treated with imidocarb dipropionate. However such an injection is painful due to tissue irritation. Further it suffers the drawbacks common to such anti-parasitics: the prevention of a build up of immunological memory, potential toxicity, and possible build up of resistance.

It has been shown that Babesiosis and Theileriosis can be controlled by vaccination with live vaccines (reviewed in: Jenkins, M. 2001, Vet Parasitol., vol. 101, p. 291-310). Such vaccines are produced by harvesting erythrocytes from infected animals. For some but not all Babesia species *in vitro* erythrocyte cultures have been developed, to increase the number of parasites. The infected erythrocytes from the animal or the cultures, also known as "stabilates", are then used to vaccinate animals.

Stabilates for Theileria are produced in a similar fashion. In fact, because the need for an effective vaccine is so high, Theileria stabilates have even been produced from the salivary glands of infected ticks.

General disadvantages of such live parasitic vaccines are that the inoculation material is largely uncontrolled, highly variable in its composition, biologically unsafe, and on the whole the process is unethical through the use of a large number of experimental animals. Additionally, Piroplasmid parasites are very unstable; they must be kept away from free oxygen or will die quickly.

suggested that the protective activity is due to the immunising capacity of antigens of the merozoite surface coat in the serum or medium, a structure that is left behind during the process of invasion of the erythrocyte (Ristic, M. and Montenegro-James, S., 1988, in: "Babesiosis of Domestic Animals and Man", Ristic, M. ed., p. 163-190, CRC Press). In addition, during *in vitro* culture a number of parasites die, thereby (internal) parasitic antigens are released into the culture medium.

Such SPA preparations are capable of inducing an immune response that, although not necessarily affecting the parasite, sufficiently reduces the clinical manifestations of infection (Schetters and Montenegro-James, S., 1995, Parasitology Today, vol. 11, p. 456-462). For instance SPA from culture supernatant of an *in vitro* culture of *Babesia canis* parasite infected erythrocytes (Pirodog®) induces immunity against homologous (but not to heterologous) challenge infection.

In general, SPA based vaccines bear the same disadvantages as the live parasitic vaccines do, in that they are largely uncharacterised, highly variable and require many precautions to be biologically safe. Additionally the production of such vaccines is very difficult to scale up, as that requires the infection, housing and harvesting from samples of experimental animals to provide parasites, erythrocytes, and/or serum.

It is an object of the invention to provide proteins and fragments thereof that can serve in effective vaccines for prevention or amelioration of infection with a Piroplasmid organism, that are well defined, safe, stable, and with a production that is easy to scale up.

It was surprisingly found now that a vaccine comprising one or more of five novel Piroplasmid proteins, or an immunogenic fragment of one or more of said proteins incorporate all these advantageous characteristics.

Many disadvantages of live parasite- and SPA vaccines can now be overcome by the use of such a Piroplasmid protein or of an immunogenic fragment of said protein in vaccines. Such a protein is highly defined, biologically safe, the product can be stabilized much better than whole live parasites, and its production can be easily scaled up

It was surprisingly found that antibodies raised against Piroplasmid proteins or immunogenic fragments of said proteins, effectively inhibited the invasion of parasites into host cells, and thereby interfered with the parasites' infection cycle. The proteins are therefore called: invasion inhibiting antigen (IIA).

The process of the invasion by a Piroplasmid parasite of its host cell is one of the critical steps in the establishment of parasitic infection. By interfering at this level through induction of antibodies that interfere with this step, the initial entry of parasites into the cells of the host is inhibited. This prevents, or at least diminishes, the level of infection or the clinical signs of disease in a host, and consequently the severity of disease. Also the further spread of the disease in the environment is halted or diminished because less ticks will become carriers when feeding on vaccinated hosts, *ergo* the infection pressure in the environment is decreased.

Piroplasmid IIA's, which can induce protective immune responses that lead to antibodies that inhibit Piroplasmid parasite invasion, can be detected in Piroplasmid parasites, in cultures of proliferating parasites, and in infected cells by specific antisera. These specific antisera recognize these IIA also in 1D and 2D Western blots of lysates of infected cells, of parasites or their cultures.

The Piroplasmid IIA's can be expressed in an expression system. Proteins, or their fragments, expressed in this way can be used to formulate a vaccine which protects mammalians from disease or its clinical signs upon infection by a Piroplasmid organism, through the induction of specific antibodies or antigen-specific lymphocytes.

Therefore the invention provides a Piroplasmid protein characterised in that said protein comprises an amino acid sequence having a similarity of at least 70%, preferably 75 %, more preferably 80, 85, 90, 92, 94, 95, 96, 97, 98, 99, or 100 % similarity in that order of preference, with the amino acid sequence depicted in SEQ ID NO: 2 or 4, or an immunogenic fragment of said protein.

The invention also provides a Piroplasmid protein characterised in that said protein comprises an amino acid sequence having a similarity of at least 70%, preferably 75 %, more preferably 80, 85, 90, 92, 94, 95, 96, 97, 98, 99, or 100 % similarity in that order of preference, with the amino acid sequence depicted in SEQ ID NO: 6 or 8, or an immunogenic fragment of said protein.

Typical examples of the Piroplasmid proteins of the invention are:

- Piroplasmid IIA number 1 from *Babesia bovis* (BIIA1) the amino acid sequence of which is presented in SEQ ID NO: 2;
- Piroplasmid IIA number 1 from *Theileria annulata* (TIIA1) the amino acid sequence of which is presented in SEQ ID NO: 4;
- Piroplasmid IIA number 2 from *B. bovis* (BIIA2) the amino acid sequence of which is presented in SEQ ID NO: 6;
- Piroplasmid IIA number 2 from *T. annulata* (TIIA2) the amino acid sequence of which is presented in SEQ ID NO: 8;
- Piroplasmid IIA number 3 from *B. bovis* (BIIA3) the amino acid sequence of which is presented in SEQ ID NO: 10.

The term "protein" is meant to incorporate a molecular chain of amino acids. A protein is not of a specific length, structure or shape and can, if required, be modified *in vivo* or *in vitro*, by, e.g. glycosylation, amidation, carboxylation, phosphorylation, or changes in spatial folding. *Inter alia*, peptides, oligopeptides and polypeptides are included within the definition of protein. A protein can be of biologic and/or of synthetic origin.

A "Piroplasmid protein" according to the invention is a protein, which is obtainable from an organism of the Piroplasmids.

Preferably the Piroplasmid protein is obtainable from an organism selected from the group consisting of the species *Babesia divergens, B. bovis, B. motasi, B. caballi, B. equi, B. canis, B. rossi, B. vogeli, B. felis, B. cati, B. ovis, B. trautmanni, B. bigemina, B. microti, B. gibsoni, Theileria annulata, T. parva, T. equi, T. felis, T. canis* and *T. sergenti.*

More preferably the Piroplasmid protein is obtainable from an organism selected from the group consisting of the species *Babesia bovis, , B. caballi, B. equi, B. canis, B. rossi, B. bigemina, Theileria annulata, T. parva* and *T. equi.*

Even more preferably, the Piroplasmid protein is obtainable from an organism selected from the group consisting of the species *Babesia bovis and Theileria annulata.*

Most preferably the Piroplasmid protein is obtainable from *Babesia bovis.*

With respect to the current taxonomic classification, the skilled person will realise this may change over time as new insights lead to reclassification into new or other taxonomic groups. However, as this does not change the protein repertoire of the organism involved, only its classification, such re-classified organisms are considered to

be within the scope of the invention. This is especially relevant for such closely related families as Babesiidae and Theileriidae. For example: *Babesia equi* was recently re-classified as *Theileria equi*.

In order to be antigenic, a fragment of a protein needs to be of a certain length; too small fragments will not be processed by antigen presenting cells to fragments that are able as such to associate with MHC molecules, which association is required for proper antigen presentation to lymphocytes. For MHC I receptor binding an antigen fragment that encompasses the epitope consists of at least 8 – 11 amino acids, and for MHC II receptor binding at least 11 – 15 amino acids (reviewed e.g. by R.N. Germain & D.H. Margulies, 1993, Annu. Rev. Immunol., vol. 11, p. 403-450, in: "The biochemistry and cell biology of antigen processing and presentation"). Protein fragments shorter than this may not be antigenic as such: they need to be coupled to a carrier, such as KLH, BSA or the like, using techniques known in the art. When coupled such short fragments may well be able to induce an immune response that is within the scope of the invention.

For the invention, an "epitope" is that part of an antigenic molecule that reacts with the antigen receptor of a T- and/or B-lymphocyte. An epitope according to the invention will therefore induce and/or activate specific T- and/or B-cells such that these cells give rise to an immune reaction that interferes with the course of an infection or disease. Thus, through such epitopes, a protein can induce antibodies and/or generate an immune response.

An "immunogenic fragment" is understood to be an epitope containing antigenic fragment of a Piroplasmid protein that has the capability to induce immune responses directed against such Piroplasmid proteins, with the provision that such antibodies are capable of interfering with the process of invasion. It will be explained below how such immunogenic fragments can be found.

An immunogenic fragment of a Piroplasmid protein according to the invention comprises at least 10 amino acids from the amino acid sequence of a Piroplasmid

For instance an immunogenic fragment of a protein of a Piroplasmid protein according to the invention is formed by a part of the protein that lacks the N-terminal signal sequence and/or the C-terminal sequence. Other fragments are for instance those comprising a specific epitope from a Piroplasmid IIA protein. Such epitopes may be determined by the methods outlined below. All such immunogenic fragments are within the scope of the invention.

Identification of immunogenic fragments and/or epitopes of a Piroplasmid protein according to the invention, can be easily performed by a variety of straightforward techniques, for instance by the so-called PEPSCAN method, or via computer algorithms that make comparisons to known fragments and/or epitopes.

The PEPSCAN method (WO 84/03564, and WO 86/06487, and H. Geysen *et al.*, Proc. Natl. Acad. Sci. USA 1984, vol. 81, p. 3998-4002, and J. of Immunol. meth. 1987, vol. 102, p. 259-274), is an easy to perform, quick and well-established method for the detection of immunologic determinants of a protein. It comprises the synthesis of a series of peptide fragments progressively overlapping the protein under study, and subsequent testing of these polypeptides with specific antibodies to the protein to identify which of these are able to bind to the antigen receptor of T- and/or B-lymphocytes. Such antibodies to the proteins according to the invention can be obtained by making polyclonal or monoclonal antibodies, by using techniques well known in the art.

The use of computer algorithms in the designation of specific protein fragments as the immunologically important epitopes on the basis of their sequential and/or structural agreement with epitopes that are known, is also a well-known technique. The determination of these regions can be based on a combination of the hydrophilicity criteria according to Hopp and Woods (Proc. Natl. Acad. Sci. USA 1981, vol. 78, p. 3824-3828), and the secondary structure aspects according to Chou and Fasman (Advances in Enzymology 1987, vol. 47, p. 45-148, and US patent 4,554,101). Immunogenic epitopes can likewise be predicted from the protein's amino acid sequence by computer with the aid of Berzofsky's amphiphilicity criterion (, Science 1987, vol. 235, p. 1059-1062 and US patent application NTIS US 07/005,885). A condensed overview of the use of these methods is found in Shan Lu (common principles: Tibtech 1991, vol. 9, p. 238-242), Lu (review: Vaccine 1992, vol. 10, p. 3-7), and Berzofsky (HIV-epitopes; 1991, The FASEB Journal, vol. 5, p. 2412-2418).

An illustration of the effectiveness of using these methods was published by H. Margalit *et al.* (, J. of Immunol. 1987, vol. 138, p. 2213-2229) who describe success rates of 75 % in the prediction of T-cell epitopes using such methods. Still further proof is the

successful prediction of the 6 antigenic peptides from BIIA1 and BIIA2, as outlined in Example 1, section 1.1.5.

Subsequently, it has to be determined if an epitope found using the methods described above is indeed capable of interfering with the process of invasion. This can however be done very quickly and easily in a simple *in vitro* invasion inhibition experiment. Such an experiment is described in Example 1.1.11.

The percentage of similarity of an amino acid sequence with a protein according to the invention must be determined by amino acid alignment to the full-length amino acid sequence of SEQ ID NO: 2, 4, 6, 8, or 10.

The percentage of similarity with a protein according to the invention must be determined with the computer program "BLAST 2 SEQUENCES" by selecting sub-program: "BlastP" (T. Tatusova & T. Madden, 1999, FEMS Microbiol. Letters, vol. 174, p. 247-250), that can be found at www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html. The comparison-matrix that is used is: "Blosum62", with the default parameters: open gap penalty: 11; extension gap penalty: 1, and gap x_dropoff: 50.

This program lists the percentage of amino acids that are identical as "Identities", and the percentage of amino acids that are similar as "Positives". "Similar" amino acids are those amino acids that are identical plus those that are equivalent; "equivalent" amino acids are described below.

It will be understood that, for a particular Piroplasmid protein, natural variations exist between the proteins associated with individual strains or species of Piroplasmids. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. Amino acid substitutions, which do not essentially alter biological and immunological activities, have been described, e.g. by Neurath *et al.* (1979, in: "The Proteins", Academic Press New York). Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, i.a. Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., 1978, "Atlas of protein sequence

(Science 1985, vol. 227, p. 1435-1441) and determining the functional similarity between proteins. Such amino acid substitutions of the exemplary embodiments of this invention, as well as variations having deletions and/or insertions are within the scope of the invention as long as the resulting proteins retain the capability of inducing immune responses that inhibit Piroplasmid parasite proliferation, for instance antibodies that inhibit Piroplasmid parasite invasion. Such variations in the amino acid sequence of a certain Piroplasmid protein according to the invention are considered as "biological- or functional homologs", and are all within the scope of the invention.

This explains why a Piroplasmid protein according to the invention, when isolated from different Piroplasmid species, may have a similarity down to 70 % with for example the amino acid sequences depicted in SEQ ID NO: 2, 4, 6, 8, or 10 while still representing the same protein with the same characteristics, in the example presented: to be able to induce antibodies that inhibit Piroplasmid parasite invasion.

When comparing Piroplasmid proteins according to the invention amongst themselves, Piroplasmid proteins according to the invention obtained from different Piroplasmid organisms typically have over 50 % amino acid similarity; when obtained from different Babesia species, such proteins typically have over 85 % amino acid similarity, and when obtained from different isolates from *B. bovis*, such proteins typically have over 95 % amino acid similarity.

The preferred way to produce the Piroplasmid proteins according to the invention is by using genetic engineering techniques and recombinant expression systems. These may comprise using nucleic acids, cDNA fragments, recombinant DNA molecules, live recombinant carriers, and/or host cells.

Therefore, another aspect of the invention relates to a nucleic acid, characterised in that said nucleic acid encodes a Piroplasmid protein according to the invention, or an immunogenic fragment of said protein.

In an embodiment the nucleic acid according to the invention comprises the nucleic acid sequence depicted in SEQ ID NO: 1, 3, 5, 7, or 9.

The term "nucleic acid" is meant to incorporate a molecular chain of desoxy- or ribonucleic acids. A nucleic acid is not of a specific length, therefore polynucleotides, genes, open reading frames (ORF's), probes, primers, linkers, spacers and adaptors,

consisting of DNA and/or RNA, are included within the definition or nucleic acid. A nucleic acid can be of biologic and/or synthetic origin. The nucleic acid may be in single stranded or double stranded form. The single strand may be in sense or anti-sense orientation. Also included within the definition are modified RNAs or DNAs. Modifications in the bases of the nucleic acid may be made, and bases such as Inosine may be incorporated. Other modifications may involve, for example, modifications of the backbone.

The term "encodes" is meant to incorporate: providing the possibility of protein expression, i.a. through transcription and/or translation when brought into the right context.

A nucleic acid according to the invention encodes a Piroplasmid protein according to the invention, or encodes an immunogenic fragment of said protein.

A nucleic acid according to the invention has a minimal length of 30 nucleotides. Preferably a nucleic acid according to the invention comprises 40, 50, 100, 250, 500, 1000, or 1500 nucleotides in that order of preference.

A nucleic acid according to the invention for instance is a nucleic acid encoding a Piroplasmid protein according to the invention that lacks the N-terminal signal sequence and/or the C-terminal sequence. Other nucleic acids may comprise a sequence encoding a specific epitope of a Piroplasmid protein. Such nucleic acids are all within the scope of the invention.

Excluded from the nucleic acids according to the invention are the following sequences:

- with regard to BIIA1 (SEQ ID NO: 1), the EST sequences:
  - B_bovis-11e05.plc
  - B_bovis-344e09.qlc
  - B_bovis-384f06.qlc
  - B_bovis-261d05.qlc
  - B_bovis-5e5.plc
  - B_bovis-[illegible].qlc

- with regard to BIIA1 (SEQ ID NO: 1), the assembled contigs:
  - Bbovis.CONTIG.1029
  - Bbovis.CONTIG.227

- With regard to BIIA2 (SEQ ID NO: 5) the EST sequences:
  - B_bovis-417g12.qlc
  - B_bovis-376a10.qlc

- with regard to TIIA2 (SEQ ID NO: 7), the assembled contig:
  - gnl|Sanger_5874|Contig1548

- with regard to TIIA1 (SEQ ID NO: 3), the assembled contig:
  - gnl|Sanger_5874|Contig1

The EST and contig sequences regarding BIIA1 and BIIA2 are available through the Internet web page: www.sanger.ac.uk/projects/b_bovis/ .

The contig sequences regarding TIIA1 and TIIA2 are available through the NCBI BLAST server by selecting Apicomplexa from the Internet page: http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi?organism=euk

The percentage of identity between nucleic acids according to the invention is determined with the computer program "BLAST 2 SEQUENCES" by selecting sub-program: "BlastN" (T. Tatusova & T. Madden, 1999, FEMS Microbiol. Letters, vol. 174, p. 247-250), that can be found at www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html. Parameters that are used are the default parameters: reward for a match: +1; penalty for a mismatch: -2; open gap penalty: 5; extension gap penalty: 2; and gap x_dropoff: 50. Unlike the output of the BlastP program described above, the BlastN program does not list similarities, only identities: the percentage of nucleotides that are identical are indicated as "Identities".

It is well known in the art, that many different nucleic acids can encode one and the same protein. This is a result of what is known in molecular biology as "wobble", or the "degeneracy of the genetic code"; when several codons or triplets of mRNA will cause the same amino acid to be attached to the chain of amino acids growing in the ribosome during translation. It is most prevalent in the second and especially the third base of each

triplet encoding an amino acid. This phenomenon can result in a heterology of about 30% for two different nucleic acids that still encode the same protein. Therefore, two nucleic acids having a nucleotide sequence identity of about 70 % can still encode one and the same protein.

Another approach for deciding if a certain nucleic acid sequence is or is not a nucleic acid sequence according to the invention, relates to the question if that certain nucleic acid sequence does hybridise under stringent conditions to any of the nucleotide sequences depicted in SEQ ID NO: 1, 3, 5, 7, and 9.

If a nucleic acid sequence hybridises under stringent conditions to the nucleotide sequence as depicted in SEQ ID NO: 1, 3, 5, 7, and 9, it is considered to be a nucleic acid sequence according to the invention.

The definition of stringent conditions follows from the formula for the melting temperature Tm of Meinkoth and Wahl (1984, Anal. Biochem., vol. 138, p. 267-284):

$$Tm = [81.5°C + 16.6(\log M) + 0.41(\%GC) - 0.61(\%formamide) - 500/L] - 1°C/1\%mismatch$$

In this formula, M is molarity of monovalent cations; %GC is the percentage of guanosine and cytosine nucleotides in the DNA; L is the length of the hybrid in base pairs, and mismatch is the lack of an identical match.

Stringent conditions are those conditions under which nucleic acid sequences or fragments thereof still hybridise, if they have a mismatch of 30 % (i.e. if they are only 70 % identical) to the nucleic acid sequence as depicted in any of the SEQ ID NO's: 1, 3, 5, 7, and 9.

Nucleic acids encoding the Piroplasmid proteins according to the invention can be obtained from member species of the Piroplasmida.

However in a more preferred embodiment, the nucleic acids encoding a Piroplasmid protein or immunogenic fragments of said protein according to the invention are characterised in that they are obtainable from an organism selected from the group consisting of the species *Babesia divergens, B. bovis, B. motasi, B. caballi, B. equi, B.*

The possibility of species being taxonomically re-classified or described as new species has been discussed above. As this does not change the organism's genome, such reclassified organisms are also within the scope of the invention.

Also within the scope of the invention are Piroplasmid proteins, immunogenic fragments of said proteins and nucleic acids encoding such Piroplasmid proteins or fragments thereof from non-mammalian Piroplasmids, due to the high conservation of the genes and proteins of the Piroplasmid proteins according to the invention. Such related proteins, or their genes may be called paralogs or orthologs.

Nucleic acids encoding a Piroplasmid protein according to the invention can be obtained, manipulated and expressed by standard molecular biology techniques that are well-known to the skilled artisan, and are explained in great detail in standard text-books like Sambrook & Russell: "Molecular cloning: a laboratory manual" (2001, Cold Spring Harbour Laboratory Press; ISBN: 0879695773). One such type of manipulations is the synthesis of a cDNA fragment from RNA, preferably from mRNA that can be isolated from parasites, or parasite- infected cells or -organisms by techniques known in the art.

Therefore, in another aspect, the invention relates to a cDNA fragment according to the invention.

The preferred method of obtaining a cDNA fragment by reverse transcription is through a polymerase chain reaction (PCR) technique. Standard techniques and protocols for performing PCR are for instance extensively described in C. Dieffenbach & G. Dveksler: "PCR primers: a laboratory manual" (1995, CSHL Press, ISBN 879694473).

In a preferred embodiment, the invention relates to a recombinant DNA molecule comprising a nucleic acid according to the invention, or a cDNA fragment according to the invention, said nucleic acid or said cDNA fragment being under the control of a functionally linked promoter.

To construct a recombinant DNA molecule according to the invention, preferably DNA plasmids are employed. Such plasmids are useful e.g. for enhancing the amount of DNA-insert, as a probe, and as tool for further manipulations. Examples of such plasmids for cloning are plasmids of the pBR, pUC, and pGEM series; all these are available from several commercial suppliers.

The nucleic acid encoding a Piroplasmid protein according to the invention or an immunogenic fragment of said protein, can be cloned into separate plasmids and be modified to obtain the desired conformation using techniques well known in the art. However they may also be combined into one construct for improved cloning or expression purposes.

Modifications to the coding sequences encoding a Piroplasmid protein according to the invention or an immunogenic fragment thereof may be performed e.g. by using restriction enzyme digestion, by site directed mutations, or by polymerase chain reaction (PCR) techniques.

For the purpose of protein purification or -detection, or improvement of expression level, additional nucleic acids may be added. This may result in the final nucleic acid comprised in the cDNA fragment, or in the recombinant DNA molecule being larger than the sequences required for encoding a Piroplasmid protein. When such additional elements are inserted in frame, these become an integral part of the Piroplasmid protein that is expressed. Such fused proteins are also within the scope of the invention

An essential requirement for the expression of a nucleic acid, cDNA fragment, or recombinant DNA molecule is that these are operably linked to a transcriptional regulatory sequence such that this is capable of controlling the transcription of the nucleic acid, cDNA, or recombinant DNA. Transcriptional regulatory sequences are well known in the art and comprise i.a. promoters and enhancers. It is obvious to those skilled in the art that the choice of a promoter extends to any eukaryotic, prokaryotic or viral promoter capable of directing gene transcription, provided that the promoter is functional in the expression system used.

In a more preferred embodiment, the invention relates to a live recombinant carrier comprising a nucleic acid according to the invention or a cDNA fragment according to the invention, said nucleic acid or said cDNA fragment being under the control of a functionally linked promoter, or a recombinant DNA molecule according to the invention.

Such live recombinant carriers (LRC's) are e.g. micro-organisms such as bacteria, parasites and viruses in which additional genetic information has been cloned. In this case [illegible] a recombinant DNA molecule encoding a Piroplasmid protein

sequence encoding a Piroplasmid protein according to the invention, or an immunogenic fragment thereof.

As an example of bacterial LRC's, attenuated *Salmonella* strains known in the art can attractively be used.

Alternatively, live recombinant carrier parasites have i.a. been described by Vermeulen, A. N. (Int. Journ. Parasitol. 1998, vol. 28, p. 1121-1130).

LRC viruses may be used as a way of transporting a nucleic acid into a target cell. Live recombinant carrier viruses are also called vector viruses. Viruses often used as vectors are Vaccinia viruses (Panicali et al. 1982, Proc. Natl. Acad. Sci. USA, vol. 79, p. 4927), Herpesviruses (EP 0473210-A2), and Retroviruses (Valerio, D. et al. 1989, in: Baum, S.J., Dicke, K.A., Lotzova, E. and Pluznik, D.H. (Eds.), "Experimental Haematology today", Springer Verlag, New York: pp. 92-99).

The technique of *in vivo* homologous recombination, well known in the art, can be used to introduce a recombinant nucleic acid according to the invention into the genome of an LRC bacterium, parasite or virus of choice, capable of inducing expression of the inserted nucleic acid, cDNA or recombinant DNA according to the invention in the host animal.

Bacterial, yeast, fungal, insect, and vertebrate cell expression systems are used as host cells for expression purposes very frequently. Such expression systems are well known in the art and generally available, e.g. commercially through Invitrogen (the Netherlands).

Therefore, in an even more preferred embodiment, the invention relates to a host cell comprising a nucleic acid according to the invention, a cDNA fragment according to the invention, said nucleic acid or said cDNA fragment being under the control of a functionally linked promoter, a recombinant DNA molecule according to the invention, or a live recombinant carrier according to the invention.

A host cell to be used for expression of a Piroplasmid protein according to the invention may be a cell of bacterial origin, e.g. from *Escherichia coli*, *Bacillus subtilis*, *Lactobacillus sp.* or *Caulobacter crescentus*, in combination with the use of bacteria-derived plasmids or bacteriophages for expressing the sequence encoding a Piroplasmid protein. The host cell may also be of eukaryotic origin, e.g. yeast-cells in combination with yeast-specific vector molecules, or higher eukaryotic cells, like insect cells (Luckow et al.,1988, Bio-technology, vol. 6, p. 47-55) in combination with vectors or recombinant baculoviruses; plant cells in combination with e.g. Ti-plasmid based vectors or plant viral

vectors (Barton, K.A. *et al.*, 1983, Cell, vol. 32, p. 1033); or mammalian cells like Hela cells, Chinese Hamster Ovary cells or Crandell-Rees feline kidney-cells, also with appropriate vectors or recombinant viruses.

Next to these expression systems, plant cell, or parasite-based expression systems are attractive expression systems. Parasite expression systems are e.g. described in the French Patent Application, publication number 2 714 074, and in US NTIS publication no. US 08/043109 (Hoffman, S. & Rogers, W., 1993). Plant cell expression systems for polypeptides for biological application are e.g. discussed in R. Fischer *et al.* (Eur. J. of Biochem. 1999, vol. 262, p. 810-816), and J. Larrick *et al.* (Biomol. Engin. 2001, vol. 18, p. 87-94).

Expression may also be performed in so-called cell-free expression systems. Such systems comprise all essential factors for expression of an appropriate recombinant nucleic acid, operably linked to a promoter that will function in that particular system. Examples are the *E. coli* lysate system (Roche, Basel, Switzerland), or the rabbit reticulocyte lysate system (Promega corp., Madison, USA).

The Piroplasmid protein according to the invention or immunogenic fragments of said protein are very well suited for the production of a vaccine. Such proteins or fragments can be obtained from parasites, or from animals or cells infected with Piroplasmid parasites. However, much more convenient is the use of the nucleic acids encoding the Piroplasmid protein according to the invention or an immunogenic fragment of said protein, in an expression system. This is followed by harvesting the proteins or fragments produced and formulating these into a protein subunit vaccine, e.g. by admixing a Piroplasmid protein according to the invention or an immunogenic fragment of said protein, and a pharmaceutically acceptable carrier.

Therefore, yet another aspect of the invention relates to a vaccine comprising a protein according to the invention or an immunogenic fragment of said protein, a nucleic acid, a cDNA fragment, a recombinant DNA molecule, a live recombinant carrier, or a host cell according to the invention, or a combination thereof, and a pharmaceutically acceptable carrier.

protective immune responses (e.g. specific antibodies or activated lymphocytes) that interfere with parasite proliferation, or the clinical signs it produces.

If such proteins or fragments do not produce the desired response on their own, they can be coupled to a carrier such as KLH, BSA or the like, using techniques known in the art.

The coupling of protein or fragments thereof can also be done to enhance or modify the immune response induced. For instance it is common practice to couple protein(-fragment)s to Tetanus toxoid to enhance the response of T-cells. Also specific effector molecules may be added, such as a toxin, to improve the killing of target cells.

Such couplings can be performed

- chemically, by coupling, conjugation or cross-linking, through dehydration, esterification, etc, of the amino acid sequences either directly or through an intermediate structure.
- physically, by coupling through capture in or on a macromolecular structure, or preferably
- by molecular biological fusion, through the combination of recombinant nucleic acid molecules which comprise fragments of nucleic acid capable of encoding each of the two, such that a single continuous expression product is finally produced.

Such molecular engineering techniques are preferred.

An alternative and efficient way of vaccination is by direct vaccination with DNA encoding the relevant antigen or epitope. Direct vaccination with DNA encoding proteins has been successful for many different proteins, as reviewed in e.g. Donnelly *et al.* (The Immunologist 1993, vol. 2, p. 20-26). For example in the field of anti-parasite vaccines, protection against e.g. *Plasmodium yoelii* has been obtained with DNA-vaccination with the *P. yoelii* circumsporozoite gene (Hoffman, S. *et al.* 1994, Vaccine, vol. 12, p. 1529-1533), and protection against *Leishmania major* has been obtained with DNA-vaccination with the *L. major* surface glycoprotein gp63 gene (Xu & Liew 1994, Vaccine, vol. 12, p. 1534-1536).

Such a DNA vaccination can be performed with a nucleic acid, a cDNA fragment, or preferably with a recombinant DNA molecule according to the invention.

Therefore, one preferred embodiment relates to a vaccine according to the invention, characterised in that it comprises a nucleic acid, a cDNA fragment, or a recombinant DNA molecule according to the invention.

Alternatively, a vaccine according to the invention can comprise live recombinant carriers as described above, capable of expressing the Piroplasmid protein according to the invention or immunogenic fragments of said protein. Such vaccines, e.g. based upon a bacterial, a parasitic or a viral carrier or vector have the advantage over subunit vaccines that they better mimic the natural way of infection by Piroplasmida. Also the presentation of the antigens by cells infected with the carriers resembles the route a Piroplasmid protein according to the invention or immunogenic fragments of said protein are presented to the immune system in a natural infection. Moreover, their self-propagation is an advantage since only low amounts of the recombinant carrier are necessary for immunisation.

Thus, another preferred embodiment relates to a vaccine according to the invention, which comprises a live recombinant carrier and a pharmaceutically acceptable carrier.

The host cells as described above can be used to express a Piroplasmid protein according to the invention or an immunogenic fragment of said protein as an expression system. After expression the proteinacious product may be harvested, but alternatively the culture medium or the complete host cells themselves may be used in a vaccine. This has the benefit of omitting purification steps, but of course requires some tolerance by the target mammalians for the media components and/or components of the host cells.

Also within the scope of the invention is a vaccine according to the invention comprising a combination of two or more types of molecules from the Piroplasmid protein according to the invention or an immunogenic fragment of said protein, or a nucleic acid, cDNA, recombinant molecule, live recombinant carrier, or host cells according to the invention. For such vaccines according to the invention the components may be combined in a single dose or in separate doses, and these may be given at the same time or sequentially.

For instance, a combination vaccination of an initial priming with a recombinant DNA plasmid carrying the coding sequence of a Piroplasmid protein, followed some time later by a booster vaccination with a Piroplasmid protein may advantageously be used.

in principle be used; preferably a dose of between 50 and 200 µg of a Piroplasmid protein or an immunogenic fragment thereof is used .

For live viral vector vaccines the dose rate per animal may range from 1 to 10^{10} pfu, preferably $10 - 10^5$ pfu are used.

A pharmaceutically acceptable carrier is understood to be a compound that does not adversely effect the health of the animal to be vaccinated, at least not to the extend that the adverse effect is worse than the effects seen when the animal would not be vaccinated. A pharmaceutically acceptable carrier can be e.g. sterile water or a sterile physiological salt solution. In a more complex form the carrier can e.g. be a buffer.

Often, a vaccine is mixed with stabilizers, e.g. to protect degradation-prone components from being degraded, to enhance the shelf-life of the vaccine, or to improve freeze-drying efficiency. Useful stabilizers are i.a. SPGA (Bovarnik *et al.* 1950, J. Bacteriology, vol. 59, p. 509), skimmed milk, gelatine, bovine serum albumin, carbohydrates e.g. sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose, proteins such as albumin or casein or degradation products thereof, and buffers, such as alkali metal phosphates.

The vaccine according to the invention may additionally comprise a so-called "vehicle". A vehicle is a compound to which the proteins, protein fragments, nucleic acids or parts thereof, cDNA's, recombinant molecules, live recombinant carriers, and/or host cells according to the invention adhere, without being covalently bound to it. Such vehicles are i.a. bio-microcapsules, micro-alginates, liposomes, macrosols, aluminium-hydroxide, -phosphate, -sulphate or -oxide, silica, Kaolin®, and Bentonite®, all known in the art.

An example is a vehicle in which the antigen is partially embedded in an immune-stimulating complex, the so-called ISCOM® (EP 109.942, EP 180.564, EP 242.380).

In addition, the vaccine according to the invention may comprise one or more suitable surface-active compounds or emulsifiers, e.g. Span® or Tween®.

Target subjects for the vaccine according to the invention are preferably mammalian, e.g. humans or mammalian animals of veterinary importance. The target may be healthy or diseased, and may be seropositive or -negative for Piroplasmidal parasites or for antibodies to Piroplasmidal parasites. The target subject can be of any age at which it is susceptible to the vaccination.

The more preferred target mammalians for the vaccine according to the invention are bovines, equines, canines, and felines.

The vaccine according to the invention can equally be used as prophylactic and as therapeutic treatment, and interferes with the establishment and/or with the progression of an infection or its clinical symptoms of disease.

Therefore one aspect of the invention relates to the use of a nucleic acid sequence according to the invention, a cDNA fragment according to the invention, a recombinant DNA molecule according to the invention, a live recombinant carrier according to the invention, or a host cell according to the invention for the manufacture of a vaccine for prophylactic or therapeutic treatment of an infection or its clinical signs caused by a Piroplasmid organism.

The vaccine according to the invention prevents or reduces the spread of Piroplasmid infection through the population or to the environment.

The vaccine according to the invention can be in several forms, e.g.: a liquid, a gel, an ointment, a powder, a tablet, or a capsule, depending on the desired method of application to the target.

Preferably the vaccine is in the form of an injectable liquid.

The vaccine according to the invention can be administered to the mammalian target according to methods known in the art. For instance by parenteral applications such as through all routes of injection into or through the skin: e.g. intramuscular, intravenous, intraperitoneal, intradermal, submucosal, or subcutaneous. Alternative routes of application that are feasible are by topical application as a drop, spray, gel or ointment to the mucosal epithelium of the eye, nose, mouth, anus, or vagina, or onto the epidermis of the outer skin at any part of the body; by spray as aerosol, or powder. Alternatively, application can be via the alimentary route, by combining with the food, feed or drinking water e.g. as a powder, a liquid, or tablet, or by administration directly into the mouth as a liquid, a gel, a tablet, or a capsule, or to the anus as a suppository.

The preferred application route is by intramuscular or by subcutaneous injection.

It goes without saying that the optimal route of application will depend on the [illegible] of the disease infection or clinical [illegible] to be prevented.

The scheme of the application of the vaccine according to the invention to the target mammalian can be in single or multiple doses, which may be given at the same time or sequentially, in a manner compatible with the dosage and formulation, and in such an amount as will be immunologically effective.

The vaccines of the invention are advantageously applied in a single yearly dose.

In a preferred embodiment, the vaccine according to the invention is characterised in that it comprises an adjuvant.

An adjuvant in general is a substance that boosts the immune response of the target in a non-specific manner. Many different adjuvants are known in the art. Examples of adjuvants are Freund's Complete and -Incomplete adjuvant, vitamin E, non-ionic block polymers and polyamines such as dextransulphate, carbopol and pyran. Also very suitable are saponins, which are the preferred adjuvants. Saponins are preferably added to the vaccine at a level between 10 and 10.000 µg/ml. Within the group of saponins, the saponin Quil A® is the more preferred adjuvant. Saponin and vaccine components may be combined in an ISCOMS® (EP 109.942, EP 180.564, EP 242.380).

Furthermore, peptides such as muramyldipeptides, dimethylglycine, tuftsin, are often used as adjuvant, and mineral oil e.g. Bayol® or Markol®, vegetable oils or emulsions thereof and DiluvacForte® can advantageously be used.

It goes without saying that other ways of adjuvating, adding vehicle compounds or diluents, emulsifying or stabilizing a vaccine are also within the scope of the invention. Such additions are for instance described in well-known handbooks such as: "Remington: the science and practice of pharmacy" (2000, Lippincot, USA, ISBN: 683306472), and: "Veterinary vaccinology" (P. Pastoret et al. ed., 1997, Elsevier, Amsterdam, ISBN 0444819681).

The vaccine according to the invention can advantageously be combined with another antigen, or with an immunoactive component. This can also be added in the form of its encoding nucleic acid.

Therefore, in a more preferred embodiment the vaccine according to the invention is characterised in that it comprises an additional immunoactive component or a nucleic acid encoding said additional immunoactive component

The additional immunoactive component(s) may be an antigen, an immune enhancing substance, and/or a vaccine; either of these may comprise an adjuvant.

The additional immunoactive component(s) when in the form of an antigen may consist of any antigenic component of human or veterinary importance. It may for instance comprise a biological or synthetic molecule such as a protein, a carbohydrate, a lipopolysacharide, a nucleic acid encoding a proteinacious antigen, or a recombinant nucleic acid molecule containing such a nucleic acid operably linked to a transcriptional regulatory sequence. Also a host cell comprising such a nucleic acid, a recombinant nucleic acid molecule, or an LRC containing such a nucleic acid, may be a way to deliver the nucleic acid or the additional immunoactive component. Alternatively it may comprise a fractionated or killed microorganism such as a parasite, bacterium or virus.

The additional immunoactive component(s) may be in the form of an immune enhancing substance e.g. a chemokine, or an immunostimulatory nucleic acid, e.g. a CpG motif. Alternatively, the vaccine according to the invention, may itself be added to a vaccine.

For instance a vaccine according to the invention can be combined with a preparation of a Babesia subunit vaccine protein, not being a Piroplasmid protein according to the invention or an immunogenic fragment of said protein, to form a combination subunit vaccine against Piroplasmidal infection or associated clinical signs of disease.

Alternatively, the vaccine according to the invention can advantageously be combined with a pharmaceutical component such as an antibiotic, a hormone, or an anti-inflammatory drug.

In an even more preferred embodiment, the vaccine according to the invention is characterised in that said additional immunoactive component or nucleic acid encoding said additional immunoactive component is obtained from an organism infective to:
canines: *Ehrlichia canis*, *Babesia gibsoni*, *B. vogeli*, *B. rossi*, *Leishmania donovani*-complex, Canine parvovirus, Canine distemper virus, *Leptospira interrogans* serovars canicola, *icterohaemorrhagiae*, *pomona*, *grippotyphosa*, *bratislava*, Canine hepatitis virus, [illegible]

coli, Enterobacter, Klebsiella, Citrobacter, Cryptosporidium, Salmonella and *Streptococcus dysgalactiae*; and to
equines: *Streptococcus equi, Streptococcus zooepidemicus, Rhodococcus equi, Corynebacterium pseudotuberculosis, Pseudomonas mallei, Actinobacillus equili* and *Pasteurella multocida*. Potomac fever agent, *Clostridium tetanii, Mycobacterium pseudomallei*, Vesicular Stomatitisvirus, Borna disease virus, Equine influenza virus, African horse sickness virus, Equine arteritis virus, Equine herpes virus 1-4, Infectious anaemia virus, Equine encephalomyelitis virus and Japanese B encephalitis virus.

The Piroplasmid protein according to the invention, or the immunogenic fragment of said protein, the nucleic acid, cDNA, recombinant molecule, live recombinant carrier, and/or the host cells according to the invention for the first time allow the generation of specific antibodies against a Piroplasmid protein, or an immunogenic fragment of said protein. This makes the vaccine according to the invention suitable as marker vaccine, as it allows the differentiation between parasite infected and -vaccinated mammalian targets, through methods known in the art.

Alternatively, these specific antibodies may be used as a vaccine themselves, for so called "passive vaccination".

Therefore another aspect of the invention relates to a vaccine, characterised in that it comprises an antibody against a protein according to the invention, or an antibody against an immunogenic fragment of said protein, or a combination thereof, and a pharmaceutically acceptable carrier.

The antibody may be of natural or synthetic origin. The antibody may be in the form of an antiserum or a purified antibody. Such purified antibodies can advantageously be obtained from an expression system.

Methods for large-scale production of antibodies according to the invention are also known in the art. Such methods rely on the cloning of (fragments of) the genetic information encoding the protein according to the invention in a filamentous phage for phage display. Such techniques are described i.a. at the "Antibody Engineering Page" under "filamentous phage display" at

http://aximt1.imt.uni-marburg.de/~rek/aepphage.html., and in review papers by Cortese, R. et al., (1994) in Trends in Biotechn. vol. 12, p. 262-267; by Clarckson, T. & Wells, J.A. (1994) in Trends in Biotechn. vol. 12, p. 173-183; Marks, J.D. et al., (1992) J. Biol. Chem. vol. 267, p. 16007-16010; Winter, G. et al., (1994) Annu. Rev. Immunol. vol. 12, p. 433-455, and by Little, M. et al., (1994) Biotechn. Adv. vol. 12, p. 539-555.

The phages are subsequently used to screen camelid expression libraries expressing camelid heavy chain antibodies. (Muyldermans, S. and Lauwereys, M., Journ. Molec. Recogn. 12, 131-140 (1999) and Ghahroudi, M.A. et al., FEBS Letters vol. 414, p. 512-526 (1997)). Cells from the library that express the desired antibodies can be replicated and can subsequently be used for large-scale expression of antibodies.

A combination in a vaccine of an antigen 'loaded' with antibodies against that antigen is known in the art as a "complex" vaccine. Such vaccines according to the invention may advantageously be used.

For reasons of e.g. stability or economy the Piroplasmid protein according to the invention or immunogenic fragments of said protein, or nucleic acids, cDNA's, recombinant molecules, live recombinant carriers, host cells or vaccines according to the invention may be freeze-dried. In general this will enable prolonged storage at temperatures above zero ° C, e.g. at 4°C.

Procedures for freeze-drying are known to persons skilled in the art; equipment for freeze-drying at different scales is available commercially.

Therefore, in a most preferred embodiment, the vaccines according to the invention are characterised in that said vaccines are in a freeze-dried form.

To reconstitute a freeze-dried vaccine, it may be suspended in a physiologically acceptable diluent. Such a diluent can e.g. be as simple as sterile water, or a physiological salt solution. In a more complex form it may be suspended in an emulsion as outlined in PCT/EP99/10178.

Still another aspect of the invention relates to a method for the preparation of a vaccine according to the invention, said method comprising the admixing of a protein according to the invention or an immunogenic fragment of said protein, a nucleic acid, a cDNA fragment, a recombinant DNA molecule, a live recombinant carrier, or a host cell according to the invention, or a combination thereof, and a pharmaceutically acceptable carrier.

fragment of said protein, or a combination thereof, and a pharmaceutically acceptable carrier

As outlined above, a vaccine obtainable by the methods according to the invention can equally be used as prophylactic and as therapeutic treatment, and will interfere both with the establishment and/or with the progression of an infection or its clinical signs of disease.

Therefore, a further aspect of the invention relates to the use of a protein according to the invention or an immunogenic fragment of said protein, for the manufacture of a vaccine for prophylactic or therapeutic treatment of an infection or its clinical signs caused by an organism of the Piroplasmida.

Again a further aspect of the invention relates to a diagnostic test for the detection of a nucleic acid associated with a Piroplasmid organism, characterised in that the test comprises a nucleic acid, said nucleic acid being at least 70 %, preferably 75 %, more preferably 80, 85, 90, 92, 94, 95, 96, 97, 98, 99, or 100 % in that order of preference, similar to the nucleic acid sequence depicted in SEQ ID NO: 1, 3, 5, 7, or 9 or a nucleic acid that is complementary to said nucleic acid, wherein either of the nucleic acids have a length of at least 15 nucleotides, preferably 17, more preferably 18, 19, 20, 24, 28, 32, 35 or 40 nucleotides, in that order of preference.

Yet a further aspect of the invention relates to a diagnostic test for the detection of antibodies against a Piroplasmid organism, characterised in that said test comprises a protein according to the invention or an immunogenic fragment of said protein, or a combination thereof.

For instance BIIA1 or BIIA2 or an immunogenic fragment of either is coupled to a solid phase carrier, this is incubated with a sample to be tested, is washed, and presence of bound antibodies is detected. Preferred diagnostic method is by Elisa.

Still a further aspect of the invention relates to a diagnostic test for the detection of antigenic material from a Piroplasmid organism, characterised in that said test comprises an antibody against a protein according to the invention or an antibody against an immunogenic fragment of said protein, or a combination thereof.

For instance antibodies against BIIA1 or BIIA2 or an immunogenic fragment of either are coupled to a solid phase carrier, this is incubated with a sample to be tested, is washed, and presence of bound protein is detected. Preferred diagnostic method is by Elisa.

The invention will now be further described with reference to the following, non-limiting, examples.

EXAMPLES

EXAMPLE I

1.1. TECHNIQUES USED

1.1.1. *B. bovis in vitro* culture

B. bovis Israel isolate (clonal line C61411) was cultured *in vitro* as previously described (Levy & Ristic 1980, Science, vol. 207, p. 1218-1220). Briefly, *B. bovis* cultures were maintained in 24-well plates (1.2 ml total volume) or in 25 cm^2 bottles (15 ml total volume) containing medium M199 (Cambrex Bioscience, Belgium), with 40% bovine serum (from an adult donor cow), 50 $\mu g ml^{-1}$ Gentamicin (Gibco BRL), 25 mM sodium bicarbonate, and bovine erythrocytes at 5% packed cell volume (PCV). Cultures were incubated at 37°C, 5% CO_2 in air, and parasitaemia was kept between 1% and 12% by daily dilution.

1.1.2. Construction of *B. bovis* genomic and cDNA library

A cDNA library was constructed from 5 µg *B. bovis* mRNA using the λZAP-cDNA® Synthesis Kit (Stratagene) according to the manufacturer's instructions. cDNA fragments of 0.5 to 4 kb were collected by gel filtration on a sepharose CL4B column and ligated into the *Eco*RI / *Xho*I site of λ uniZAP-XR Express vector. Giga pack III Gold was used for packaging into phage particles followed by transformation of *Escherichia coli* XL-1 Blue MRF'cells. 1.2×10^8 plaques were obtained of which an amplified library was made.

Single-pass sequence runs were performed on 15000 cDNA clones that were automatically picked at random from the plated cDNA library to establish an EST dataset. From this EST dataset a database consisting of 12892 high quality sequences (476 bp average length) was constructed.

For constructing the genomic library, 600 µg of *B. bovis* DNA was partially digested with EcoRI (150 units or 250 units) for 1 h at 37°C. The digested DNA was size fractionated on a Sepharose CL-4B column. Fragments of 0.5 kb to 8 kb were ligated into the EcoRI site of λ-ZAPII-Express, packaged using Gigapack III Gold Packaging extract and transformed in *E. coli* XL1-Blue MRF'competent cells. 2.5×10^6 plaques were obtained of which an amplified library was made.

The cDNA libraries were screened with a probe produced through PCR with primers specific for BIIA1 or for BIIA2.

1.1.3. screening of *B. bovis* genomic and cDNA library for the genes for BIIA1 and BIIA2

The *B. bovis* genomic and cDNA libraries were screened to isolate clones for the genes of BIIA1 and BIIA2 with a specific probe made by PCR. Specific primers used were:

for the BIIA1 gene:

primer 1: 5'- CCACGGCTCTGGAATCTATGTC -3' (SEQ ID NO: 11)

primer 2: 5'- CAAAAGGATACCTATATTTGGTAC -3' (SEQ ID NO: 12),

and for the BIIA2 gene:

primer 3: 5'- TGTGGTAGATGAATCTGCTAGTATATC -3' (SEQ ID NO: 13)

Primer 4: 5'- CTATGCCACGGCATTCAGCAACATTTA -3' (SEQ ID NO: 14)

Both primer pairs were used to amplify a fragment from a clone from the EST database of *B. bovis*, by PCR in a 50 µl volume containing 0.2 mM dNTP, 20 pmol/µl of each primer, 100 ng *B. bovis* total genomic DNA and 0.5 U Taq DNA polymerase in standard buffer (Promega). Amplification was performed for 30 cycles with the conditions for the BIIA1 probe at: 92°C for 30 s, 58°C for 30 s, at 72°C for 30 s, and for the BIIA2 probe at: 95°C for 1 min, 58°C for 1 min, at 72°C for 10 min. These cycles were preceded by initial denaturation for 3 min at 95°C and a final elongation at 72°C for 10 min.

Both probes were purified from agarose gel and labelled with 50 µCi ^{32}P-dATP (3000 Ci/mmol), using a Random Primer labelling kit (Roche). In total 4.10^6 cDNA and 4.10^5 genomic DNA library plaques were screened by standard procedures (Sambrook & Russell, supra) for cloning the BIIA1 cDNA; whereas 5.10^5 cDNA and an equal number of genomic DNA library plaques were screened for cloning the BIIA2 cDNA. After 2 cycles of plaque purification all clones were *in vivo* excised for isolation of the phagemids inserts as described in the manufacturer's instructions (Stratagene) and sequenced on both strands, using automated cycle sequencing with the dye terminator method (ABI PRISM® dye terminator kit, Pharmacia).

1.1.4. Expression of recombinant BIIA1 in *E. coli*

The clones of BIIA1 en BIIA2 were subcloned by PCR from the pCR2.1 cloning plasmids.

The primers used for subcloning BIIA1 were:

primer 5: 5'- CCCGGATCCATGCAGTTACATAACAAA -3' (SEQ ID NO: 15)

primer 6: 5'- GGGAAGCTTCTGAGCAAAGGAAATAGG -3' (SEQ ID NO: 16)

These primers for BIIA1 introduced a *Bam*HI RESTRICTION ENZYME site prior to base 1 (numbered from the first base of the initiation codon) and a *Hind*III site after base 1504.

The primers used for subcloning BIIA2 were:

primer 7: 5'- CCCGAATTCGTGGTAGATGAATCTGCT -3' (SEQ ID NO: 17)

primer 8: 5'- CCCGTCGACTGCCTCGCCCCAAATGTTGT -3' (SEQ ID NO: 18)

These primers for BIIA2 introduced an *Eco RI* site, and a *Sal I* site.

After PCR (30 cycles of 1min 94°C, 1 min 55°C, 1min 72°C), the fragments were gel purified, annealed to pET-32a vector and used for transformation in *E. coli* NovaBlue® strain. Plasmids containing the appropriate insert were used to transform in expression host strains, BL21 (DE3). Fusion proteins with thioredoxin were obtained with maximal yield after induction with 1 mM of isopropyl-β-D thiogalactosidase (IPTG) for 4 hr at 37°C as shown by analysis of total cell samples at 0 and 4 hr after induction. Bacterial pellets were boiled at 95°C in SDS-polyacrylamide (SDS-PAGE) sample buffer containing 2% (v/v) β-mercaptoethanol, run on 10% SDS-PAGE minigels, and Coomassie Brilliant Blue stained to confirm expression (Figures 1 and 2).

1.1.5. Peptide selection and generation of monospecific antiserum

After the BIIA1 and BIIA2 genes were completely sequenced, peptides were selected from computer-translated sequences, for induction of specific polyclonal antibodies through immunisation of test animals.

The sequence analysis program Protean of DNA Star® was used to select peptide regions that have a good surface probability and contained charged alpha amphiphathic regions.

Peptides selected from BIIA1 (SEQ ID NO: 2) were:

peptide 1: aa numbers 46-60: cysteine-AFHKEPNNRRLTKRS,
peptide 2: aa numbers 395-409: cysteine-RGVGMNWATYDKDSG,
peptide 3: aa numbers 453-467: cysteine-YVEPRAKNTNKYLDV.

Peptides selected from BIIA2 (SEQ ID NO: 6) were:
peptide 4: aa numbers 255-269: cysteine-PGKRTRALLDLRMIE ,
peptide 5: aa numbers 424-439: cysteine-RVGNTDEEHNHRKDMD,
peptide 6: aa numbers 547-561: cysteine-VYDDHPEESENTGIN.

After the synthesis of the peptides, they were coupled to a carrier protein: Maleimide activated keyhole limpet haemocyanin (KLH) (Pierce; 77605) according to the manufacturer's instructions. The peptide-carrier conjugate was used to generate rabbit polyclonal antisera.

For that purpose three groups of NZW-rabbits (each group contained 2 rabbits) were immunized five times subcutaneously with a 3-week interval between consecutive immunizations. Before the immunisation blood serum was collected of each rabbit, which was used as negative control. Each rabbit was injected with 250 µg peptide coupled to KLH that was taken up in an equal volume of adjuvant Stimune® (Id-dlo). Total volume that was injected in each rabbit was 1000 µl. Sera were tested periodically for reactivity by ELISA. Plasmaforeses were done one week after the last immunization and sera were collected.

1.1.6. ELISA

The antibody response was evaluated by ELISA. Ninety-six-well microtiter plates were coated with 150 ng of either peptide 1 or peptide 2 per well, incubated 30 min at 37°C, blocked for 1 h with PBS/BSA. Consecutive dilutions (1:50 to 1:50.000) of individual rabbit sera were incubated for 1 h at 37 °C. The plates were washed, and 1:2000 diluted swine anti rabbit HRP-conjugated secondary antibody was incubated fore 1h. The plates were washed and developed for 45 min with ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)]-peroxidase substrate (Roche biochemicals). The OD_{405} was recorded, and [illegible]

1.1.7. Immunofluorescence assay

The recognition of *B. bovis* merozoites by anti-sera against peptides from BIIA1 and BIIA2 was tested by indirect immunofluorescence assay (IFA). Thin blood smears were fixed with chilled methanol cells. Primary incubation with polyclonal rabbit anti-BIIA1 (1:40) or polyclonal mouse anti-BIIA1 (1:5 to 1:160) for 30 min was followed by three wash steps of 5 min. Slides were incubated with 1:80 goat anti-rabbit immunoglobulin G (IgG) fluorescein isothiocyanate- labelled antibodies (Nordic) for 30 min. The slides were washed again, and vectashield® solution (Vector laboratories) was applied, objects were covered with a cover-glass and visualized on a UV fluorescence microscope with FITC filters (450-480/ 515-565 nm). IFA titres were determined as the last serum dilution with a positive recognition of the parasite compared to the negative pre-immune serum diluted 1:5.

1.1.8. Preparation of total merozoite protein extracts and proteins solubilised upon invasion

800 µl samples of merozoites, prepared as described above for *in vitro* invasion, were partially separated from erythrocyte ghosts by filtration over 1.2 µM polypropylene prefilters (Millipore, AN1202500). Filtered merozoites were pooled and washed twice in 20 volumes of PBS containing 25 mM sodium bicarbonate (pH8.0) followed by centrifugation at 2000 g for 20 min at 4°C. After the second wash the pellet was resuspended in an equal volume of PBS (pH 8.0) and divided in aliquots of 200 µl that were centrifuged (10000 *g*, 5 min at 4°C) and stored as 100 µl cell pellets(2×10^9 merozoites) at -20°C after removal of supernatant. Frozen merozoite pellets were thawed just before use and lysed, reduced and alkylated by using a Proteoprep® membrane extraction kit (Sigma) according the manufacturer's instructions and finally obtained in1.7 ml of buffer compatible with direct application on SDS-polyacrylamide gels or isoelectrofocussing (IEF) strips. Insoluble material was removed by centrifugation at 16,000×g for 3 min at 4°C. Protein concentration was determined by the Bradford method (Anal. Biochem. 1976, vol. 72, p. 248-254). As the extracts contained considerable amounts of erythrocyte proteins, control extracts were prepared in the same way but starting with a culture of non-infected erythrocytes.

Proteins solubilised upon invasion were obtained by gently removing the overlaying buffer after 1 h of *in vitro* invasion as described above. The samples were centrifuged (2000 *g*, 10 min, 4 °C) after which the pellet (which was invisible) was discarded and the supernatant centrifuged again at high speed for removal of membrane

fragments (20 min, 12000 *g*, 4°C). The final supernatant was dialysed (Pierce; Snakeskin®pleated dialysis tubing, 68035) overnight against 10 mM $KHPO_4$, pH 7.5. Residual haemoglobin was removed batchwise by incubating 50 ml of the dialysed supernatant with 6.5 ml DEAE sepharose fast flow (Amersham Biosciences) equilibrated in dialysis buffer for 90 min at 4°C on a rotating platform. The suspension was centrifuged for 5 min at 3000 *g* at 4°C after which the DEAE sepharose was washed 4 times by addition of 50 ml of dialysis buffer followed by centrifugation for 5 min at 3000*g* at 4°C. Bound proteins were eluted by addition of 6 ml of elution buffer (350 mM KCl, 10 mM $KHPO_4$, pH 7.5) and incubation for 5 min followed by centrifugation for 5 min at 3000*g* at 4°C. The supernatant was concentrated and desalted over 10-kDa filters (YM-10, Millipore).

1.1.9. SDS-polyacrylamide electrophoresis and Western blotting

Proteins were resolved in the presence or absence of β-mercaptoethanol and were separated on a 10% SDS-PAGE and electrophoretically transferred to an Immobilon™-P membrane (Millipore). The blot was blocked with 5% skimmed milk diluted in 0.5% Tween 20® containing phosphate-buffered saline (PBST) for 1h at 37°C. An appropriate dilution (1:500) of primary antibody in 2% skimmed milk in PBST was incubated for 1 h overnight. The blot was washed with PBST and then incubated with a 1:10.000 dilution of anti-rabbit- horseradish peroxidase (HRP)-conjugated secondary antibody (DAKO) for 1 h at 37°C. After being washed with PBST, the blot was developed with TMB MB substrate kit (Lucron Bioproducts b.v.; KPL 50-77-00) or with enhanced chemoluminescence (ECL)+ (Amersham; RPN2132).

1.1.10. Iso-electric focusing

Total merozoite extract, invasion supernatant, and BIIA1 protein samples were resuspended in rehydration solution (7M urea, 2 M thiourea, 4% CHAPS, 2% carrier ampholyte mixture pH 4-7NL (IPG buffer and 20 mM DTT). BIIA2 protein samples were separated in the first dimension using carrier ampholyte mixture pH 3-10NL IEF [illegible]

90 min during which the voltage rose to 3500 V followed by continued focusing at 3500 V for a total of 35-40 KVh, IPGPhor™).

Following isoelectric focussing, the proteins were reduced and bound to SDS by equilibrating each strip for 15 min in 10 ml of SDS equilibration buffer (50mM Tris, 6M urea, 2% SDS, 30% glycerol, pH 8.8) containing 30 mM DTT (added fresh before use). A second equilibration step in SDS equilibration buffer containing 2.5% iodoacetamide (also freshly added) instead of dithiotreitol was performed in order to prevent protein reoxidation and to minimise reactions of cysteine residues.

The second-dimensional SDS gel electrophoresis gel was carried out in a Hoefer SE600 system. Silver staining was used to visualise proteins after 2-DE. Images of the gels were acquired using LabScan® v3.0 software on a Umax flatbed scanner and were analysed using ImageMaster® 2D v3.01 software (Amersham Biotech).
For immune blotting, proteins on 7 cm strips were separated on a 10% SDS-PAGE gel or 13 cm strips were separated on 2-D protein gel and transferred to an Immobilon™-P membrane (Millipore; IPVH00010). The procedure followed for two-dimensional blots was the same as that for the 1-D blots.

1.1.11. B. bovis *in vitro* invasion assay

Invasion was performed as described previously (Fransen et al. 2003, Microbes Infect. vol. 5, p. 365-372), with slight modifications. *B. bovis* infected red blood cells at 6 to 8% parasitaemia, were centrifuged at 2000 *g*, 10 min, 15°C, and resuspended in an equal volume of VyMs buffer (Vega & Martinez, see Fransen, supra). 800 µl samples were submitted to five intermittent (10 seconds, at 0°C in between pulses) high voltage pulses (2.5 kV, 200Ω, 25 µF) in 4 mm BioRad cuvettes (165-2088) using a BioRad Gene Pulser® with pulse controller.

8 ml of PBS containing 25 mM sodiumbicarbonate (pH 8.0, 20°C) was added to each 800 µl sample followed by centrifugation (1800 *g*) for 10 min at 15°C. A second, identical wash was performed except that centrifugation was done at 1300 *g* after which the merozoite pellet was resuspended in 800 µl PBS containing 25 mM sodiumbicarbonate (pH 8.0, 20°C). Invasion was initiated by addition of 1 volume of resuspended merozoites to 9 volumes of suspended bovine erythrocytes (5.5% PCV in PBS pH 8.0 containing 25 mM sodiumbicarbonate, pre-incubated for 30 min at 37°C in CO_2 in air) and was performed in 24-well plates (final volume 1.2 ml), in 25-cm^2 flasks (15 ml) or in 80 cm^2 flasks (50 ml) at 37°C, 5% CO_2 in air. Giemsa-stained slides were

prepared after 1 h and parasitisised erythrocytes out of a total of 5000 erythrocytes were counted.

1.1.12. *In vitro* inhibition of invasion by polyclonal rabbit antisera

200 µl of *B. bovis* merozoites, liberated by high voltage pulsing and resuspended in PBS containing 25 mM sodiumbicarbonate (pH 8.0) as described above, were incubated with 40 µl of rabbit antisera for 1 h at 20°C. After 1 h, 960 µl of suspended bovine erythrocytes (6.25% PCV in PBS pH 8.0 containing 25 mM sodiumbicarbonate, pre-incubated for 30 min at 37°C in CO_2 in air) were added, followed by 1 h of incubation after which Giemsa-stained slides were prepared and counted to determine the level of invasion. The rabbit antisera used were raised against synthetic peptides derived from the BIIA1 and BIA2 amino acid sequence and a control serum raised against an unrelated control peptide (YAGRLFSKRTAATAYKLQ). Peptides had been linked to keyhole limpet haemocyanin (KLH) prior to immunization. Pre-immune sera were also included in the test.

1.2. Results of Example 1

1.2.1. Identification and cloning of a full length cDNA encoding BIIA1 and BIIA2

Probing the *B. bovis* cDNA library with PCR probes (350 bp for BIIA1 and 450 bp for BIA2), resulted in the cloning and sequencing of a 2181 bp cDNA for BIIA1 and of 2385 bp for BIIA2. Both contained an open reading frame and a 3' non-coding region terminating in a polyA-tail. To determine the 5' capped end of the full-length mRNA's, total mRNA was dephosphorylated after which the 5' caps, which are left intact, were removed by tobacco acid pyrophosphatase followed by ligation of a specific RNA oligonucleotide. Subsequently, nested PCR on first strand cDNA allowed the cloning and sequencing of a fragment representing the 5' end of the *B. bovis* mRNA for BIIA1 and for BIIA2.

Translation by computer of the 1815 bp ORF of BIIA1 predicted a 67.2 kDa; translation of the 1965 bp ORF for BIIA2 predicted a 65.6 kDa protein.

cells (Figures 1 and 2). Polyacrylamide gel electrophoresis of total cell lysates obtained before (lane 1) and after (lane 2) induction with IPTG identified the recombinant fusion product for BIIA1 and for BIIA2. Rec BIIA1 and BIIA2 both are recognized by all three immune sera (lanes 5, 8, 11) and not by pre-immune sera (lanes 6, 9, 12) on immuno-blots. Immune recognition was specific for the BIIA part of the fusion product as a control protein, a recombinant fusion product of *B. bovis* rab5 (lane3, Asp-5 to Lys-208, GenBank Acc. No.: 324137.1) expressed in PET32a was not recognized (lanes 7, 10, 13) by these sera. Also, immune recognition was peptide specific and not due to antibodies induced by the KLH carrier protein used for immunization as antiserum raised against a KLH-linked synthetic peptide unrelated to BIIA1 or BIIA2 did not recognize the BIIA1 recombinant fusion product (lane 13).

1.2.3. Immunofluorescence microscopy

To localize the BIIA proteins in the parasite, immunofluorescence studies using rabbit antisera against the six KLH-linked peptides of BIIA1 and BIIA2 were performed on *B. bovis in vitro* cultures attached to glass slides by methanol fixation (Figures 3 and 4). Incubation with pre-immune sera (panels A, C, E) did not result in any specific staining of parasites above a background signal of faint fluorescence derived from infected as well as non-infected erythrocytes. In contrast, immune sera resulted in specific staining of parasites in any microscope field examined (panels B, D, F). Fluorescent parasites were detectable with antisera against all three peptides at a dilution of 1:5. Although intra-erythrocytic *B. bovis* parasites and free merozoites are small (~1 by 2 µm) a maximal magnification allowed a clear visualization of the staining pattern.

1.2.4. Inhibition of *in vitro* invasion by peptide-specific antisera

A *B. bovis in vitro* invasion assay, allowing the study of the invasion of erythrocytes by free merozoites in a protein free buffer within a time span of 1 h, was used to assess the effect of antisera directed against the 6 peptides derived from different domains of BIIA1 and BIIA2. Free merozoites were pre-incubated for 1 h at 20°C with the anti-peptide antisera and with the control serum directed against a non-related peptide after which invasion was started by the addition of erythrocytes. All antisera against the BIIA peptides gave rise to significant inhibition of invasion whereas pre-immune sera and control antiserum had no significant effect on invasion efficiency (Figures 5 and 6). For BIIA1, the strongest effect of 65% ± 10 inhibition of invasion was observed by the antiserum directed

against peptide1; for BIIA2, the strongest effect of 70% ± 10 inhibition of invasion was observed by the antiserum directed against peptide 4.

1.2.5. Mapping BIIA proteins on 2D-gels

To determine whether BIIA1 and BIIA2 become exposed in the medium as soluble proteins during invasion of erythrocytes, thus constituting part of the SPA mentioned above, immunoblotting of invasion supernatants was performed. BIIA1 and BIIA2 were localized on two-dimensional immunoblots. 50 µg of concentrated invasion supernatant was separated by isoelectrofocussing followed by electrophoresis on SDS-polyacrylamide gels. Proteins were blotted on PVDF membranes. Excised parts of the membranes (45 to 90 kDa) were incubated with anti-BIIA1 peptide antisera against peptides 1 or 3 (Figure 7, panels A and C respectively) as well as with anti-BIIA2 peptide antisera against peptides 4 and 6 (Figure 8, panels A and C respectively). For both proteins, antibodies against peptides 1 and 4, were bound to the same specific spots (arrows) in addition to aspecific staining of proteins that were also present on control blots. These had been prepared from supernatants of uninfected red blood cells (RBC) prepared under identical conditions but in absence of merozoites (Figure 7 and 8, panels B and D). Spots localized by immunoblotting were subsequently matched to a silver-stained 2-D-protein gel of a similar sample that was obtained from a parallel experiment in which use was made of parasites that were metabolically labelled with ^{35}S-Met prior to invasion. Figure 9 displays the pattern obtained after exposure to film showing exclusively proteins of *B. bovis* as erythrocyte proteins have not incorporated label. By using imaging software, the spots detected by immunoblotting with anti-BIIA1-peptide antisera could be matched to a row of ~70 kDa spots on the autoradiograph and on the silverstained gel (see arrows on Figure 9). BIIA2 is represented by spots of minor intensity indicating a lower abundance of the native protein.

LEGEND TO THE FIGURES

Figure 1:

Lane 1: pET-BIIA1 before induction with IPTG.
Lane 2: pET-BIIA1 4 h after induction with IPTG.
Lane 3: pET-Rab5 4 h after induction.
Lanes 4, 5, 6 incubated with anti-peptide 1;
Lanes 7, 8, 9 incubated with anti-peptide 2;
Lanes 10, 11, 12 incubated with anti-peptide 3.
Lanes 4, 7, 10 contain pET-BIIA1 4 h after induction incubated with pre-immune sera;
Lanes 5, 8, 11 the same as in lanes 4, 7, and 10, but incubated with immune sera.
Lanes 6, 9, 12 contain pET-Rab5 4 h after induction incubated with immune sera.
Lane 13: pET-BIIA1 4h after induction and incubated with antiserum again KLH-linked peptide unrelated to *B. bovis*.

Figure 2:

Lane 1: pET-BIIA2 before induction with IPTG.
Lane 2: pET-BIIA2 4 h after induction with IPTG.
Lane 3: pET-Rab5 4 h after induction.
Lanes 4, 5, 6 incubated with anti-peptide 4;
Lanes 7, 8, 9 incubated with anti-peptide 5;
Lanes 10, 11, 12 incubated with anti-peptide 6.
Lanes 4, 7, 10 contain pET-BIIA2 4 h after induction incubated with pre-immune sera of rabbits;
Lanes 5, 8, 11 the same as in lanes 4, 7, and 10, but incubated with immune sera.
Lanes 6, 9, 12 contain pET-Rab5 4 h after induction incubated with immune sera.
Lane 13 contains pET-BIIA2 4h after induction and incubated with antiserum again KLH-linked peptide unrelated to B. bovis.

Figure 3:

Panels A, C and E display methanol-fixed *in vitro* cultures of *B. bovis* incubated with pre-immune rabbit antisera against peptides 1, 2 and 3 of BIIA1 respectively. Panels B, D, F are similar to A, C and E but incubated with the corresponding immune sera.

Figure 4:

Panels A, C and E display methanol-fixed *in vitro* cultures of B. bovis incubated with pre-immune rabbit antisera against peptide 4, 5 and 6 of BIIA2 respectively. Panels B, D, F are similar to A, C and E but incubated with the corresponding immune sera.

Figure 5:

Control columns represent a pre-incubation with antiserum against a non-related peptide that gave no inhibition. Antisera (open bars) as well as pre-immune rabbit sera (black bars) against peptides 1, 2 and 3 of BIIA1 were tested twice in triplo.

Figure 6:

Control columns represent a pre-incubation with antiserum against a non-related peptide that gave no inhibition. Antisera (open bars) as well as pre-immune sera (black bars) against peptides 4, 5 and 6 of BIIA2 were tested twice in triplo.

Figure 7:

Panels A and C: 2D-immunoblots with immune serum against BIIA1 peptides 1 and 3 respectively. Panels B and D: 2D-immunoblots with pre-immune serum of rabbits immunized with peptides 1 and 3 of BIIA1 respectively. Arrows indicate spots specific for antisera against peptide 1 as well as peptide 3.

Figure 8:

Panels A and C: 2D-immunoblots with immune serum against BIIA2 peptides 4 and 6 respectively. Panels B and D: 2D-immunoblots with pre-immune serum of rabbits immunized with peptide 4 and 6 of BIIA2 respectively. Arrows indicate spots specific for antisera against peptide 4 as well as peptide 6.

Figure 9:

Autoradiograph of a 2D gel as used for the immunoblots presented in figures 7 and 8, displaying only B. bovis derived proteins that were labelled with 35S-Met by metabolic labelling prior to invasion. Arrows indicate the spots that have been identified as BIIA1 and BIIA2 immunoblots shown in figure 7 and/or 8 respectively.

CLAIMS:

1. Piroplasmid protein, characterised in that said protein comprises an amino acid sequence having a similarity of at least 70% with the amino acid sequence depicted in SEQ ID NO: 2 or 4, or an immunogenic fragment of said protein.

2. Piroplasmid protein, characterised in that said protein comprises an amino acid sequence having a similarity of at least 70% with the amino acid sequence depicted in SEQ ID NO: 6 or 8, or an immunogenic fragment of said protein.

3. Piroplasmid protein, characterised in that said protein comprises an amino acid sequence having a similarity of at least 70% with the amino acid sequence depicted in SEQ ID NO: 10, or an immunogenic fragment of said protein.

4. Nucleic acid, characterised in that said nucleic acid encodes a protein according to claim 1, or an immunogenic fragment of said protein.

5. Nucleic acid, characterised in that said nucleic acid encodes a protein according to claim 2, or an immunogenic fragment of said protein.

6. Nucleic acid, characterised in that said nucleic acid encodes a protein according to claim 3, or an immunogenic fragment of said protein.

7. cDNA fragment comprising a nucleic acid according to one or more of the claims 4 - 6.

8. Recombinant DNA molecule comprising a nucleic acid according to one or more of the claims 4 - 6 or a cDNA fragment according to claim 7, said nucleic acid or said cDNA fragment being under the control of a functionally linked promoter.

9. Live recombinant carrier comprising a nucleic acid according to one or more of the claims 4 - 6, a cDNA fragment according to claim 7, said nucleic acid or said cDNA fragment being under the control of a functionally linked promoter, or a recombinant DNA molecule according to claim 8.

10. Host cell comprising a nucleic acid according to one or more of the claims 4 - 6, a cDNA fragment according to claim 7, said nucleic acid or said cDNA fragment being under the control of a functionally linked promoter, a recombinant DNA molecule according to claim 8 or a live recombinant carrier according to claim 9.

11. Vaccine comprising a protein according to one or more of the claims 1 - 3 or an immunogenic fragment of said protein, a nucleic acid according to one or more of the claims 4 - 6, a cDNA fragment according to claim 7, a recombinant DNA molecule according to claim 8, a live recombinant carrier according to claim 9, or a host cell according to claim 10, or a combination thereof, and a pharmaceutically acceptable carrier.

12. Vaccine according to claim 11, characterised in that said vaccine comprises an adjuvant.

13. Vaccine according to one or more of the claims 11 - 12, characterised in that said vaccine comprises an additional immunoactive component or a nucleic acid encoding said additional immunoactive component.

14. Vaccine, characterised in that said vaccine comprises an antibody against a protein according to one or more of the claims 1 – 3 or an antibody against an immunogenic fragment of said protein, or a combination thereof, and a pharmaceutically acceptable carrier.

15. Method for the preparation of a vaccine according to claim 11, said method comprising the admixing of a protein according to one or more of the claims 1 - 3, or an immunogenic fragment of said protein, a nucleic acid according to one or more of the claims 4 - 6, a cDNA fragment according to claim 7, a recombinant DNA molecule according to claim 8, a live recombinant carrier according to claim 9, or a host cell according to claim 10, or a combination thereof, and a pharmaceutically acceptable carrier.

17. Use of a nucleic acid sequence according to one or more of the claims 4 - 6, a cDNA fragment according to claim 7, a recombinant DNA molecule according to claim 8, a live recombinant carrier according to claim 9, or a host cell according to claim 10 for the manufacture of a vaccine for prophylactic or therapeutic treatment of an infection or its clinical signs caused by a Piroplasmid organism.

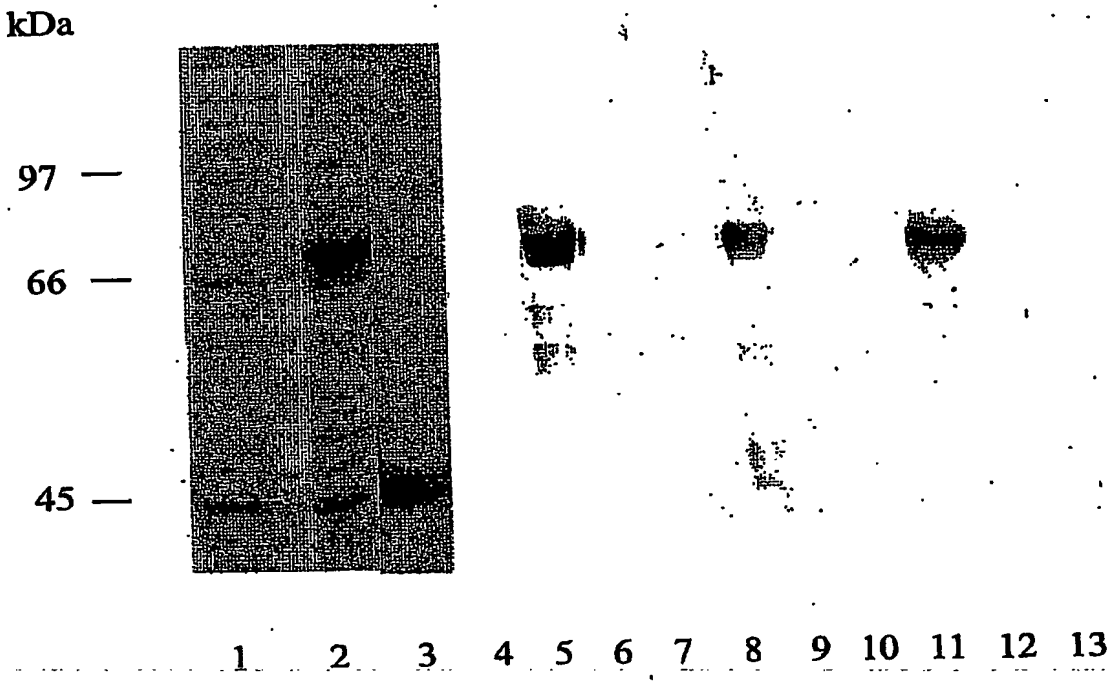
18. Diagnostic test for the detection of a nucleic acid associated with a Piroplasmid organism, characterised in that the test comprises a nucleic acid, said nucleic acid being at least 70 % similar to the nucleic acid sequence depicted in SEQ ID NO: 1, 3, 5, 7, or 9 or a nucleic acid that is complementary to said nucleic acid, wherein either of the nucleic acids have a length of at least 15 nucleotides.

19. Diagnostic test for the detection of antibodies against a Piroplasmid organism, characterised in that said test comprises a protein according to one or more of the claims 1 - 3, or an immunogenic fragment of said protein, or a combination thereof.

20. Diagnostic test for the detection of antigenic material from a Piroplasmid organism, characterised in that said test comprises an antibody against a protein according to one or more of the claims 1 - 3 or an antibody against an immunogenic fragment of said protein, or a combination thereof.

FIGURES

Figure 1



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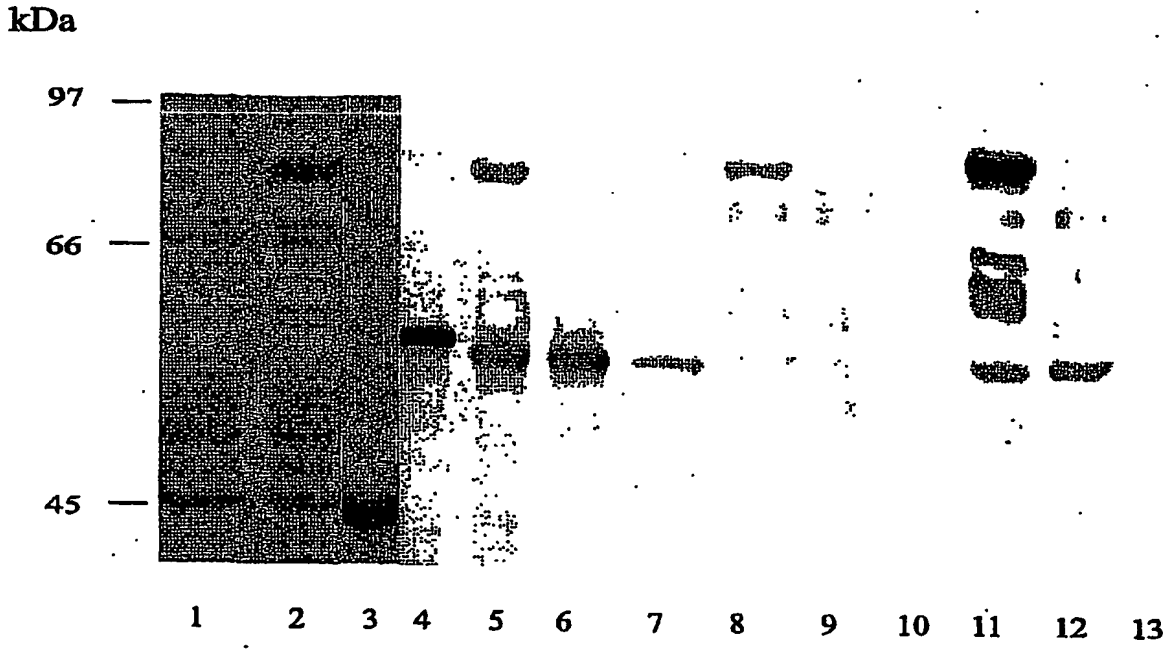

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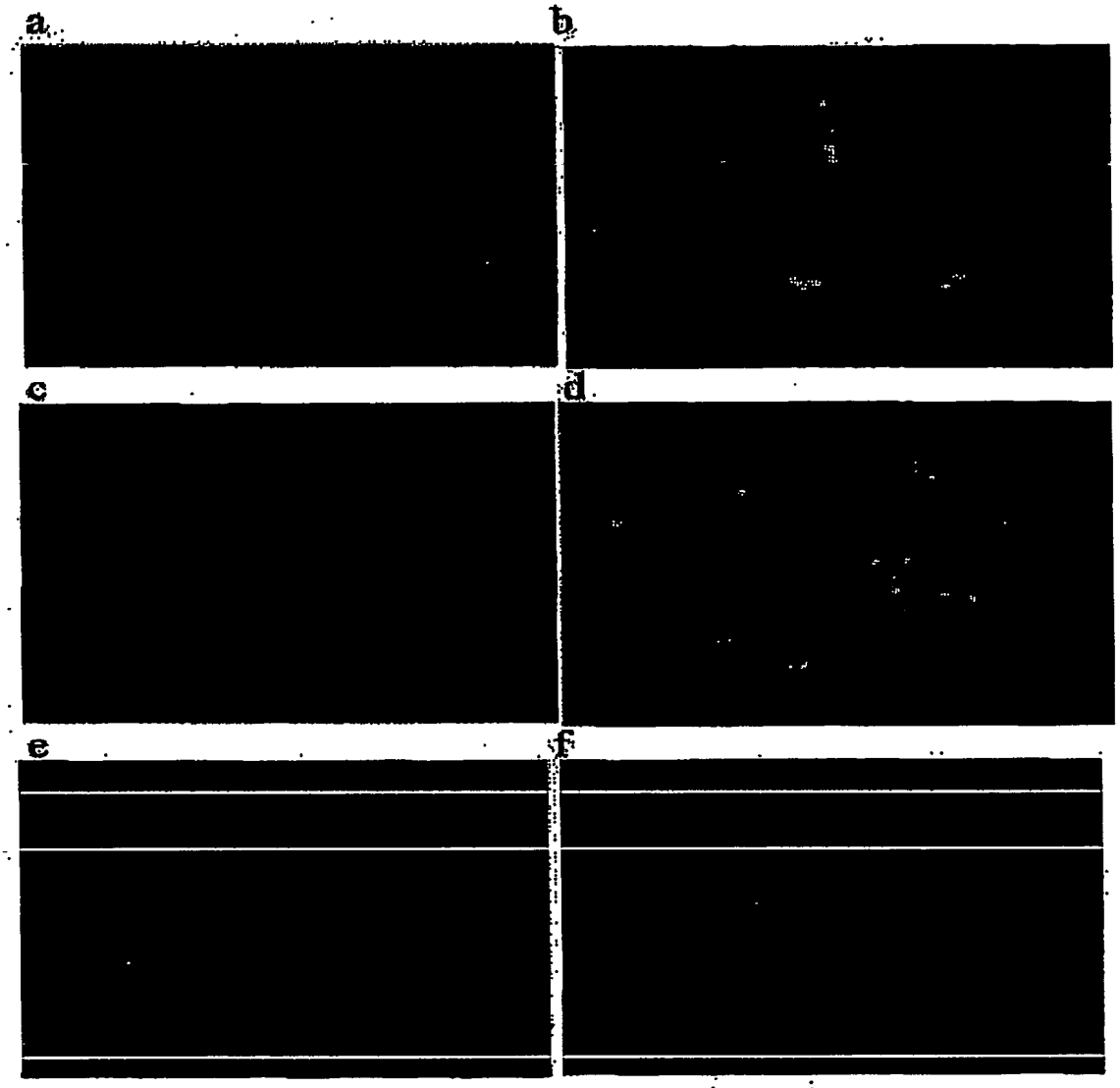

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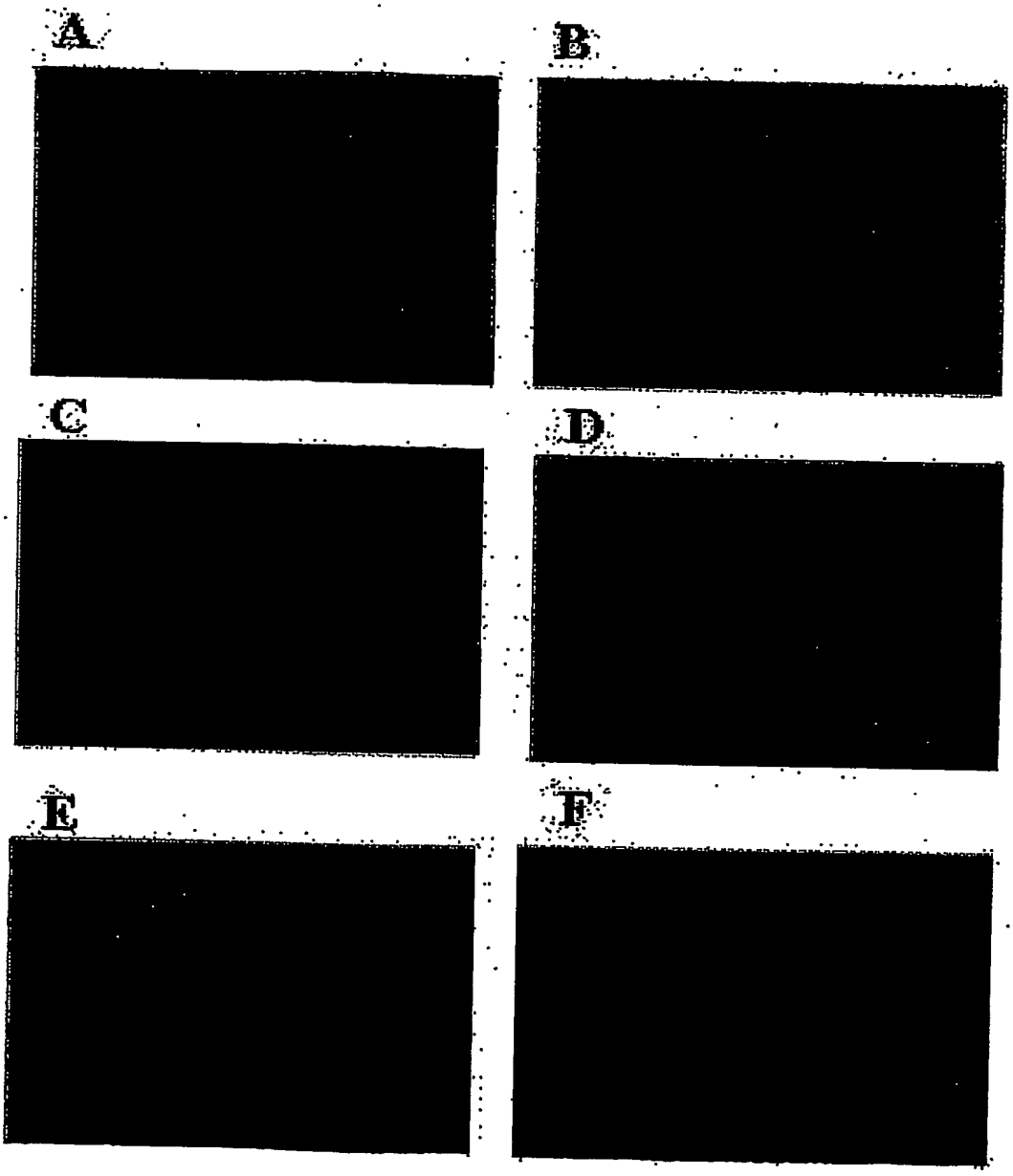

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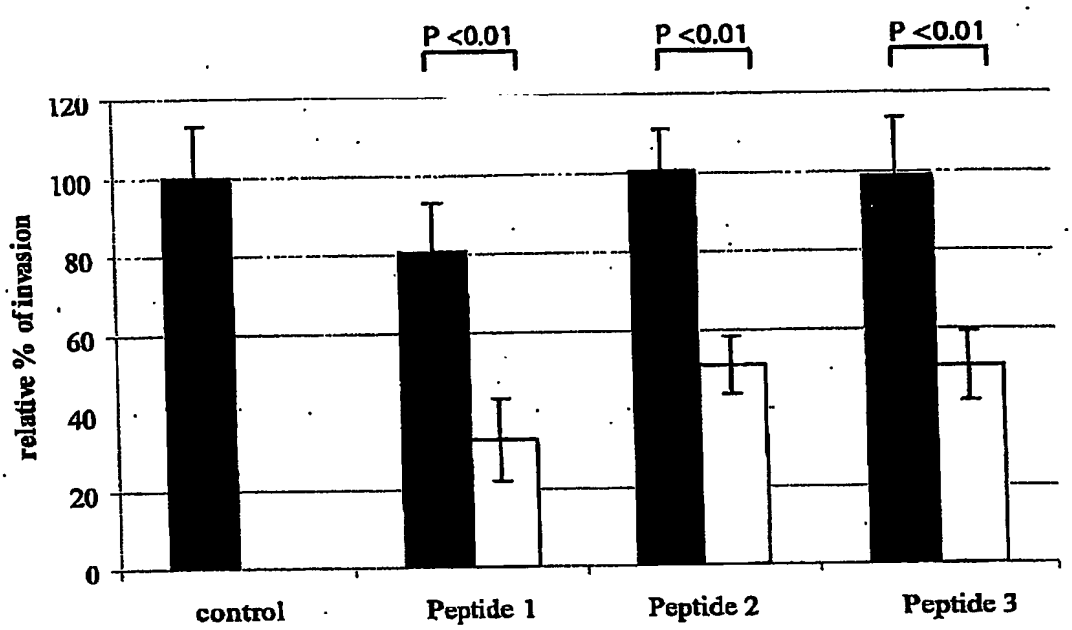
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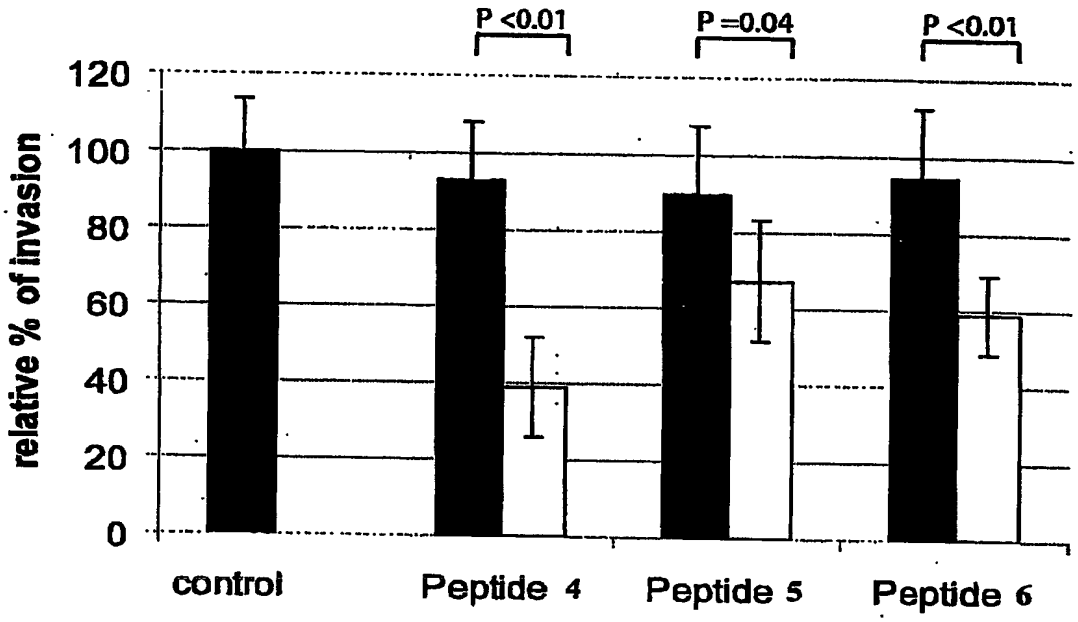
Figure 6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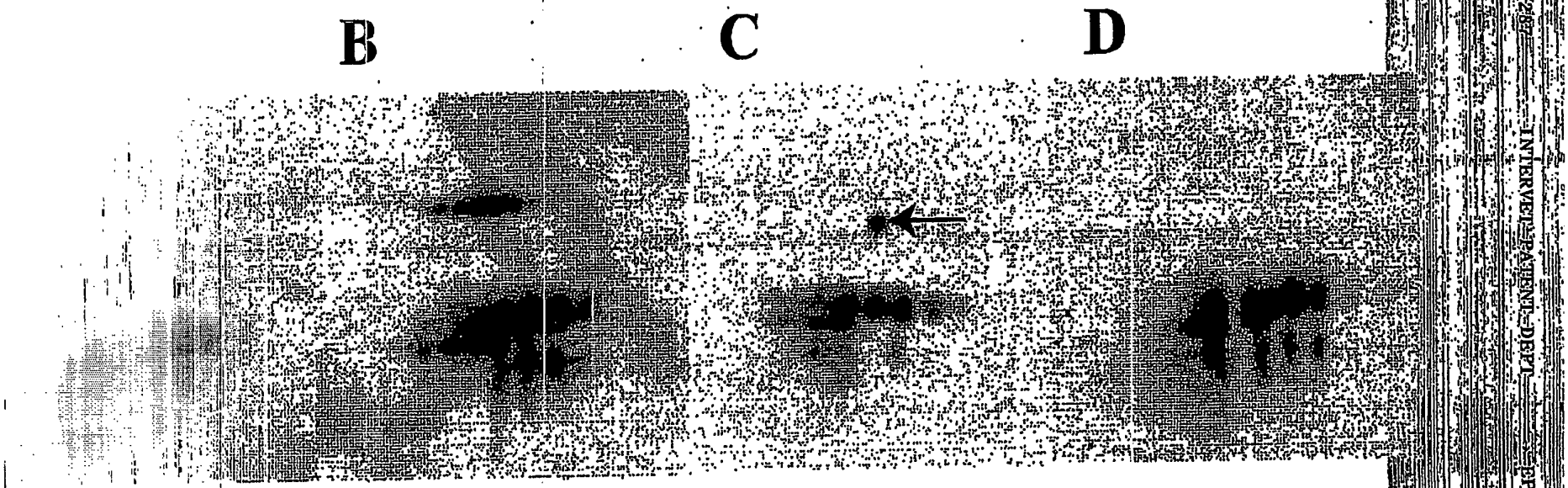


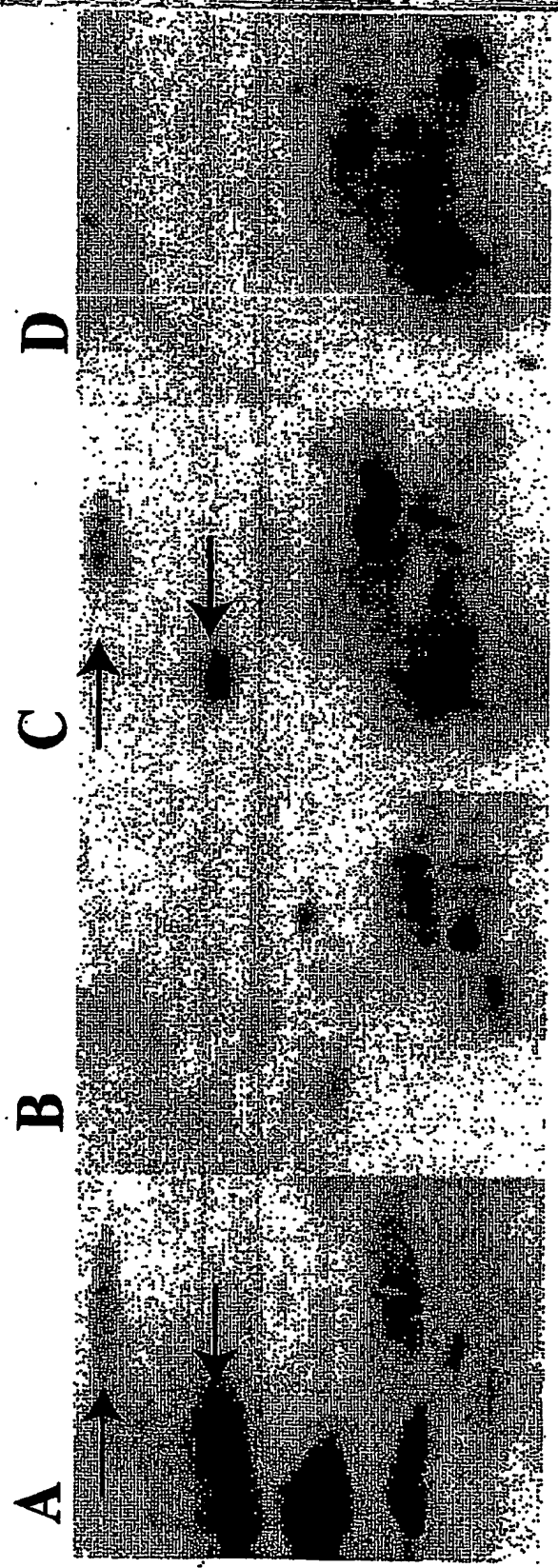
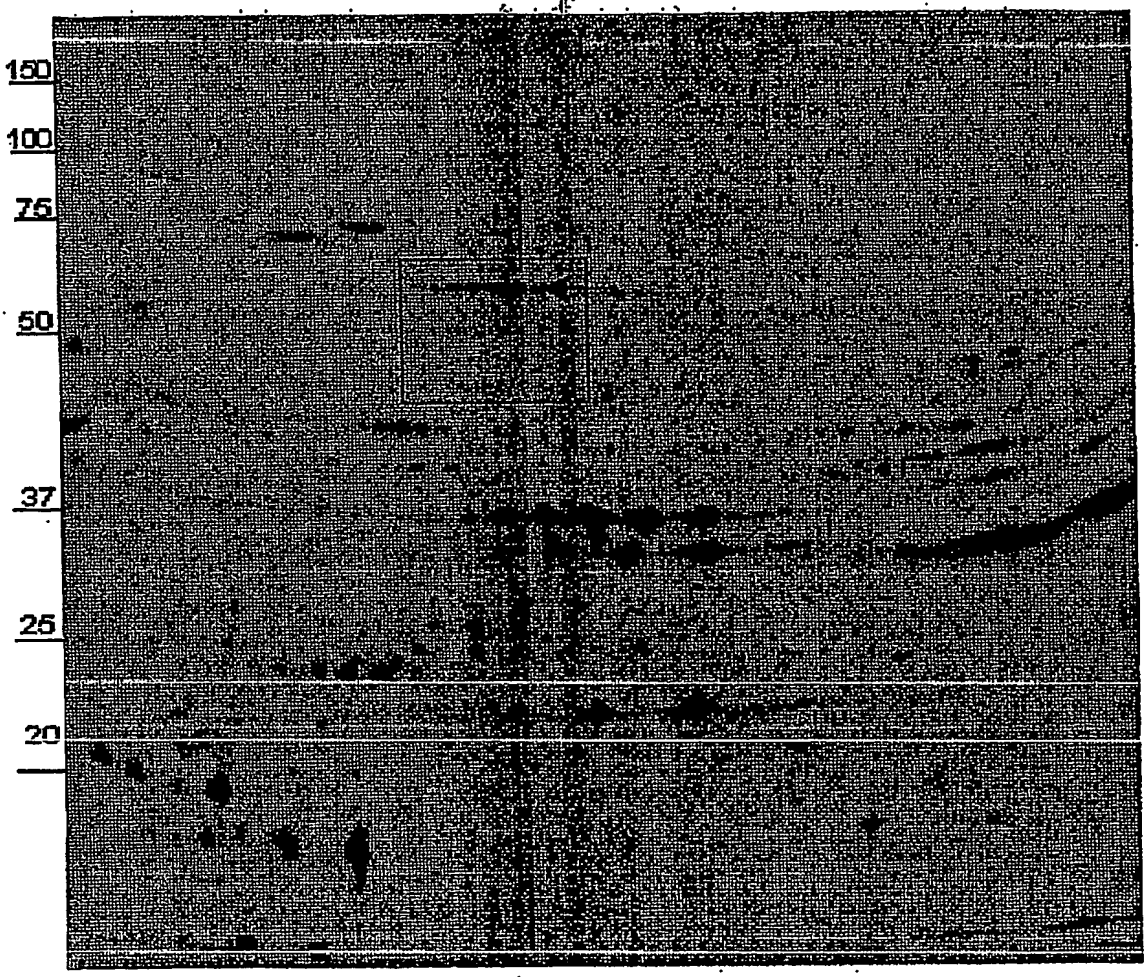


Figure 8

Figure 9



ABSTRACT

The invention relates to a Piroplasmid protein or an immunogenic fragment of said protein, and to a nucleic acid encoding said Piroplasmid protein or said immunogenic fragment. Furthermore, the invention relates to cDNA fragments, recombinant DNA molecules and live recombinant carriers comprising said nucleic acid. Also the invention relates to host cells comprising said cDNA fragments, recombinant DNA molecules and live recombinant carriers. Finally, the invention relates to vaccines comprising a Piroplasmid protein or an immunogenic fragment of said protein, to methods for the preparation of such vaccines, to the use of such proteins or fragments for vaccine purposes, and to diagnostic tests.

SEQUENCE LISTING

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    210                 215                 220

tca gac agg agt acc aaa tat agg tat cct ttt gtt ttt gac agt gat      720
Ser Asp Arg Ser Thr Lys Tyr Arg Tyr Pro Phe Val Phe Asp Ser Asp
225                 230                 235                 240

aac cag atg tgt tac ata ctg tac tct gcc ata caa tac aac caa gga      768
Asn Gln Met Cys Tyr Ile Leu Tyr Ser Ala Ile Gln Tyr Asn Gln Gly
                245                 250                 255

aat agg tat tgt gac aac gat ggt agc tcc gaa gat ggt aca agc tct      816
Asn Arg Tyr Cys Asp Asn Asp Gly Ser Ser Glu Asp Gly Thr Ser Ser
            260                 265                 270

ttg ctt tgc atg aaa cct tac aag agc gct gag gat gca cac tta tac      864
Leu Leu Cys Met Lys Pro Tyr Lys Ser Ala Glu Asp Ala His Leu Tyr
        275                 280                 285

tac ggt tct gcg aaa gtt gac ccc gat tgg gaa gaa aat tgt ccc atg      912
Tyr Gly Ser Ala Lys Val Asp Pro Asp Trp Glu Glu Asn Cys Pro Met
    290                 295                 300

cac ccg gta agg gat gcc att ttt ggt aaa tgg tct ggt ggc tct tgt      960
His Pro Val Arg Asp Ala Ile Phe Gly Lys Trp Ser Gly Gly Ser Cys
305                 310                 315                 320

gtt gcc att gct cct gca ttc caa gaa tat gcc aac agc act gaa gac     1008
Val Ala Ile Ala Pro Ala Phe Gln Glu Tyr Ala Asn Ser Thr Glu Asp
                325                 330                 335

tgt gca gcc att tta ttc gat aac tct gca act gac ttg aat atc gaa     1056
Cys Ala Ala Ile Leu Phe Asp Asn Ser Ala Thr Asp Leu Asn Ile Glu
            340                 345                 350

gct gtt aac gaa gat ttt aat gaa ctt aaa gaa ttg acc gat ggg ctt     1104
Ala Val Asn Glu Asp Phe Asn Glu Leu Lys Glu Leu Thr Asp Gly Leu
        355                 360                 365

aaa aga ttg aac atg tcg aag gtt gca aac gct att ttt tct ccc ctc     1152
Lys Arg Leu Asn Met Ser Lys Val Ala Asn Ala Ile Phe Ser Pro Leu
    370                 375                 380
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tcc aat gtt gca ggt acc agt cga att tca cgt ggt gtg ggt atg aac      1200
Ser Asn Val Ala Gly Thr Ser Arg Ile Ser Arg Gly Val Gly Met Asn
385                 390                 395                 400

tgg gct aca tac gat aaa gat tct ggt atg tgt gct ctc att aac gaa      1248
Trp Ala Thr Tyr Asp Lys Asp Ser Gly Met Cys Ala Leu Ile Asn Glu
                405                 410                 415

aca cct aac tgc ttg atc ttg aac gcg gga agc att gct ctc acg gct      1296
Thr Pro Asn Cys Leu Ile Leu Asn Ala Gly Ser Ile Ala Leu Thr Ala
            420                 425                 430

ata ggt tca cct ctc gag tat gac gct gtt aac tat cct tgc cac atc      1344
Ile Gly Ser Pro Leu Glu Tyr Asp Ala Val Asn Tyr Pro Cys His Ile
        435                 440                 445

gac acc aat ggt tac gtt gag cca cgt gca aag aat acc aac aaa tac      1392
Asp Thr Asn Gly Tyr Val Glu Pro Arg Ala Lys Asn Thr Asn Lys Tyr
    450                 455                 460

ctt gat gtt cct ttc gag gtc aca act gct ttg agc atg aag aca cta      1440
Leu Asp Val Pro Phe Glu Val Thr Thr Ala Leu Ser Met Lys Thr Leu
465                 470                 475                 480

aaa tgc gat gcc tat gtt cac acc aag tac tct gac agt tgt ggt acc      1488
Lys Cys Asp Ala Tyr Val His Thr Lys Tyr Ser Asp Ser Cys Gly Thr
                485                 490                 495

tat ttc ctt tgc tca gac gtc aaa cct aac tgg ttc att agg ttc tta      1536
Tyr Phe Leu Cys Ser Asp Val Lys Pro Asn Trp Phe Ile Arg Phe Leu
            500                 505                 510

cac atg atc gga ctc tac aac aca aag cgt atc gta ata ttc gtg tgc      1584
His Met Ile Gly Leu Tyr Asn Thr Lys Arg Ile Val Ile Phe Val Cys
        515                 520                 525

tgt acc act acc gcc atc gtt ctc act atc tgg ata tgg aaa cga ttc      1632
Cys Thr Thr Thr Ala Ile Val Leu Thr Ile Trp Ile Trp Lys Arg Phe
    530                 535                 540

atc aag gct aag aaa gag ccg gcc cct cca agt ttc gac aaa tac cta      1680
Ile Lys Ala Lys Lys Glu Pro Ala Pro Pro Ser Phe Asp Lys Tyr Leu
545                 550                 555                 560

agc aac tat gat tat gat aca acc cta gat gcc gac aac gaa acg gaa      1728
Ser Asn Tyr Asp Tyr Asp Thr Thr Leu Asp Ala Asp Asn Glu Thr Glu
                565                 570                 575

cag cgt ttg gat tcc tct gct tat agc tgg gga gag gct gta caa aga      1776
Gln Arg Leu Asp Ser Ser Ala Tyr Ser Trp Gly Glu Ala Val Gln Arg
            580                 585                 590

[illegible]                                                         1812
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Lys Arg Met Leu Cys Met Ala Leu Val Pro Val Ile Leu Ser Ser Phe
20 25 30

Phe Ala Glu Asp Ala Leu Ala Ser Asn Ser Thr Leu Phe Ala Phe His
35 40 45

Lys Glu Pro Asn Asn Arg Arg Leu Thr Lys Arg Ser Ser Arg Gly Gln
50 55 60

Leu Leu Asn Ser Arg Arg Gly Ser Asp Asp Ala Ser Glu Ser Ser Asp
65 70 75 80

Arg Tyr Pro Gly Arg Ser Gly Gly Ser Lys Asn Ser Ser Gln Ser Pro
85 90 95

Trp Ile Lys Tyr Met Gln Lys Phe Asp Ile Pro Arg Asn His Gly Ser
100 105 110

Gly Ile Tyr Val Asp Leu Gly Gly Tyr Glu Ser Val Gly Ser Lys Ser
115 120 125

Tyr Arg Met Pro Val Gly Lys Cys Pro Val Val Gly Lys Ile Ile Asp
130 135 140

Leu Gly Asn Gly Ala Asp Phe Leu Asp Pro Ile Ser Ser Asp Asp Pro
145 150 155 160

Ser Tyr Arg Gly Leu Ala Phe Pro Glu Thr Ala Val Asp Ser Asn Ile
165 170 175

Pro Thr Gln Pro Lys Thr Arg Gly Ser Ser Ser Ala Ser Ala Ala Lys
180 185 190

Leu Ser Pro Val Ser Ala Lys Asp Leu Arg Arg Trp Gly Tyr Glu Gly
195 200 205

Asn Asp Val Ala Asn Cys Ser Glu Tyr Ala Ser Asn Leu Ile Pro Ala
210 215 220

Ser Asp Arg Ser Thr Lys Tyr Arg Tyr Pro Phe Val Phe Asp Ser Asp
225 230 235 240

Asn Gln Met Cys Tyr Ile Leu Tyr Ser Ala Ile Gln Tyr Asn Gln Gly
245 250 255

Asn Arg Tyr Cys Asp Asn Asp Gly Ser Ser Glu Asp Gly Thr Ser Ser
260 265 270

Leu Leu Cys Met Lys Pro Tyr Lys Ser Ala Glu Asp Ala His Leu Tyr
275 280 285

Tyr Gly Ser Ala Lys Val Asp Pro Asp Trp Glu Glu Asn Cys Pro Met
290 295 300

His Pro Val Arg Asp Ala Ile Phe Gly Lys Trp Ser Gly Gly Ser Cys
305 310 315 320

Val Ala Ile Ala Pro Ala Phe Gln Glu Tyr Ala Asn Ser Thr Glu Asp
325 330 335

Cys Ala Ala Ile Leu Phe Asp Asn Ser Ala Thr Asp Leu Asn Ile Glu
340 345 350

Ala Val Asn Glu Asp Phe Asn Glu Leu Lys Glu Leu Thr Asp Gly Leu
355 360 365

Lys Arg Leu Asn Met Ser Lys Val Ala Asn Ala Ile Phe Ser Pro Leu
370 375 380

Ser Asn Val Ala Gly Thr Ser Arg Ile Ser Arg Gly Val Gly Met Asn
385 390 395 400

Trp Ala Thr Tyr Asp Lys Asp Ser Gly Met Cys Ala Leu Ile Asn Glu
405 410 415

Thr Pro Asn Cys Leu Ile Leu Asn Ala Gly Ser Ile Ala Leu Thr Ala
420 425 430

Leu Asp Val Pro Phe Glu Val Thr Thr Ala Leu Ser Met Lys Thr Leu
465 470 475 480

Lys Cys Asp Ala Tyr Val His Thr Lys Tyr Ser Asp Ser Cys Gly Thr
485 490 495

Tyr Phe Leu Cys Ser Asp Val Lys Pro Asn Trp Phe Ile Arg Phe Leu
500 505 510

His Met Ile Gly Leu Tyr Asn Thr Lys Arg Ile Val Ile Phe Val Cys
515 520 525

Cys Thr Thr Thr Ala Ile Val Leu Thr Ile Trp Ile Trp Lys Arg Phe
530 535 540

Ile Lys Ala Lys Lys Glu Pro Ala Pro Pro Ser Phe Asp Lys Tyr Leu
545 550 555 560

Ser Asn Tyr Asp Tyr Asp Thr Thr Leu Asp Ala Asp Asn Glu Thr Glu
565 570 575

Gln Arg Leu Asp Ser Ser Ala Tyr Ser Trp Gly Glu Ala Val Gln Arg
580 585 590

Pro Ser Asp Val Thr Pro Val Lys Leu Ser Lys Ile Asn
595 600 605

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Met Lys Lys Ile Gly Leu Lys Ile Arg Ala Gln Lys Asp Lys Leu Asn
1               5                   10                  15

cct gtg tta gga agc aac tct gac cct tcg gaa gag tat gat tca ttc      96
Pro Val Leu Gly Ser Asn Ser Asp Pro Ser Glu Glu Tyr Asp Ser Phe
            20                  25                  30

cag caa aat gtt ttc act cat caa cca acc caa cta cac aaa tct cat     144
Gln Gln Asn Val Phe Thr His Gln Pro Thr Gln Leu His Lys Ser His
        35                  40                  45
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cac tac att aca cac cag aaa aaa acc agc caa cac atc gac gat tta      192
His Tyr Ile Thr His Gln Lys Lys Thr Ser Gln His Ile Asp Asp Leu
    50                  55                  60

aat ttt tat aat gga aaa ttt aat caa aag agc aga att ggt cca ggg      240
Asn Phe Tyr Asn Gly Lys Phe Asn Gln Lys Ser Arg Ile Gly Pro Gly
65                  70                  75                  80

aag gta gta aat aac agt agg aat ctg gta gaa ggt gaa aca cta tct      288
Lys Val Val Asn Asn Ser Arg Asn Leu Val Glu Gly Glu Thr Leu Ser
                85                  90                  95

aag gat gac aat aaa aca aaa tct aaa ata aag tca aaa aca gca tca      336
Lys Asp Asp Asn Lys Thr Lys Ser Lys Ile Lys Ser Lys Thr Ala Ser
            100                 105                 110

att tta cct aga ctt tta aaa tct tta tca ttt tta gct gtt tta ggg      384
Ile Leu Pro Arg Leu Leu Lys Ser Leu Ser Phe Leu Ala Val Leu Gly
        115                 120                 125

tca att aat tca ttt tca tta gca tta gag gaa cct ttt act caa cac      432
Ser Ile Asn Ser Phe Ser Leu Ala Leu Glu Glu Pro Phe Thr Gln His
    130                 135                 140

act tct aac cga acg ccc ttt gaa gta tca tta att caa agc aac agc      480
Thr Ser Asn Arg Thr Pro Phe Glu Val Ser Leu Ile Gln Ser Asn Ser
145                 150                 155                 160

agt tta tcg cct att cat aat tct tca act caa aat tca agt cat cac      528
Ser Leu Ser Pro Ile His Asn Ser Ser Thr Gln Asn Ser Ser His His
                165                 170                 175

aac ggt ttt agt ggt agt acc gtt aat aat acc tca tta ata gag aca      576
Asn Gly Phe Ser Gly Ser Thr Val Asn Asn Thr Ser Leu Ile Glu Thr
            180                 185                 190

agg aat aac gta tta aac aga aca cta ggt aga ttc gga tca ttt ttg      624
Arg Asn Asn Val Leu Asn Arg Thr Leu Gly Arg Phe Gly Ser Phe Leu
        195                 200                 205

caa tca gga ttg ata agc agt aga gca gac aaa aag aag cgg tct ggt      672
Gln Ser Gly Leu Ile Ser Ser Arg Ala Asp Lys Lys Lys Arg Ser Gly
    210                 215                 220

atg aat aga aga ggc cct aag ggg aag aaa ggg aag gga gga gaa gac      720
Met Asn Arg Arg Gly Pro Lys Gly Lys Lys Gly Lys Gly Gly Glu Asp
225                 230                 235                 240

gaa gaa aag agg aac aag tgg acc gat ttc atg gca aag ttt gat atc      768
Glu Glu Lys Arg Asn Lys Trp Thr Asp Phe Met Ala Lys Phe Asp Ile
                245                 250                 255
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gta ggt aag gca atc ata ctc gag aat gga gct gat ttt ttg agc agc      912
Val Gly Lys Ala Ile Ile Leu Glu Asn Gly Ala Asp Phe Leu Ser Ser
    290                 295                 300

ata acc cat cat gac ccc aag gag aga ggg ctg ggg ttc cct gct aca      960
Ile Thr His His Asp Pro Lys Glu Arg Gly Leu Gly Phe Pro Ala Thr
305                 310                 315                 320

aaa gtt gcc tca aat tca tca aaa ctg gac atg gag aac cag ctc tta     1008
Lys Val Ala Ser Asn Ser Ser Lys Leu Asp Met Glu Asn Gln Leu Leu
                325                 330                 335

tca cca att agt gct cag gtc cta agg agc tgg aat tat aaa cac gaa     1056
Ser Pro Ile Ser Ala Gln Val Leu Arg Ser Trp Asn Tyr Lys His Glu
            340                 345                 350

tca gat tta agt aat tgt gct gag tat tcg aga aac att gtt ccg ggc     1104
Ser Asp Leu Ser Asn Cys Ala Glu Tyr Ser Arg Asn Ile Val Pro Gly
        355                 360                 365

agt aat cgt aat tca aag tat cgt tac ccg ttt gta tat gat gag tct     1152
Ser Asn Arg Asn Ser Lys Tyr Arg Tyr Pro Phe Val Tyr Asp Glu Ser
    370                 375                 380

gag aag ctt tgt tat att tta tat agt ccc atg caa tat aat cag ggc     1200
Glu Lys Leu Cys Tyr Ile Leu Tyr Ser Pro Met Gln Tyr Asn Gln Gly
385                 390                 395                 400

gta aag tac tgt gac caa gac tct ccg gac gaa gga act agc agt tta     1248
Val Lys Tyr Cys Asp Gln Asp Ser Pro Asp Glu Gly Thr Ser Ser Leu
                405                 410                 415

gct tgt atg tac ccg gat aag agc aag gag gat tca cac cta ttt tac     1296
Ala Cys Met Tyr Pro Asp Lys Ser Lys Glu Asp Ser His Leu Phe Tyr
            420                 425                 430

gga acc agc ggt ctt cac atg gac tgg cct gta gtt tgc cca gtt tac     1344
Gly Thr Ser Gly Leu His Met Asp Trp Pro Val Val Cys Pro Val Tyr
        435                 440                 445

cct att aga gat tcg att ttt gga tcc tac gac gac caa aag gac gaa     1392
Pro Ile Arg Asp Ser Ile Phe Gly Ser Tyr Asp Asp Gln Lys Asp Glu
    450                 455                 460

tgt gtt cca att gag ccg ata ttt gag gag gag gct gaa gat tat gag     1440
Cys Val Pro Ile Glu Pro Ile Phe Glu Glu Glu Ala Glu Asp Tyr Glu
465                 470                 475                 480

gca tgt gcc aag ata att ttc gag tat tct cca agt gat gtt gat att     1488
Ala Cys Ala Lys Ile Ile Phe Glu Tyr Ser Pro Ser Asp Val Asp Ile
                485                 490                 495

agc aca aat aac cag aag ctt tca gac gtc gac ctt tac aag gag gcg     1536
Ser Thr Asn Asn Gln Lys Leu Ser Asp Val Asp Leu Tyr Lys Glu Ala
            500                 505                 510

atg aat aat gga aag ctg agc act gct ctt tca att atg ttt gct cct     1584
Met Asn Asn Gly Lys Leu Ser Thr Ala Leu Ser Ile Met Phe Ala Pro
        515                 520                 525
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agg tac tct gag gat cgt ccg atc tat act aaa ggt gtc ggt ata aac      1632
Arg Tyr Ser Glu Asp Arg Pro Ile Tyr Thr Lys Gly Val Gly Ile Asn
    530                 535                 540

tgg gct aca tac tcc gtc gag gaa aag aaa tgt aac att ctc gac gtt      1680
Trp Ala Thr Tyr Ser Val Glu Glu Lys Lys Cys Asn Ile Leu Asp Val
545                 550                 555                 560

gtt ccc agc tgt ctt att ata agt aac ggc cac tat gcc ctt aca agt      1728
Val Pro Ser Cys Leu Ile Ile Ser Asn Gly His Tyr Ala Leu Thr Ser
                565                 570                 575

ctc agc tca ccc aat gaa gag gat gct ata aat tac ccc tgc gat atc      1776
Leu Ser Ser Pro Asn Glu Glu Asp Ala Ile Asn Tyr Pro Cys Asp Ile
            580                 585                 590

gtt cag ggc aag ggg ttt ttg aag aac cca aac ggt gga aaa aag aat      1824
Val Gln Gly Lys Gly Phe Leu Lys Asn Pro Asn Gly Gly Lys Lys Asn
        595                 600                 605

gct cag gaa ccg ccc aag gaa cct gaa cct gaa gaa cct aag aag gag      1872
Ala Gln Glu Pro Pro Lys Glu Pro Glu Pro Glu Glu Pro Lys Lys Glu
    610                 615                 620

ggt gct gaa aac aaa ccc aaa gag aaa ggt aaa tct gag aaa aag aat      1920
Gly Ala Glu Asn Lys Pro Lys Glu Lys Gly Lys Ser Glu Lys Lys Asn
625                 630                 635                 640

gaa aaa tct atg cct tca gga cca ttc acg cca tac act agc ttg aag      1968
Glu Lys Ser Met Pro Ser Gly Pro Phe Thr Pro Tyr Thr Ser Leu Lys
                645                 650                 655

aag gag ggt ttc gag tgc agt aaa tac act gtt gag cgg gtg aac aaa      2016
Lys Glu Gly Phe Glu Cys Ser Lys Tyr Thr Val Glu Arg Val Asn Lys
            660                 665                 670

agc tgc ggc gtt tac tat gaa tgc tca gaa acg cct gta tta ttt acc      2064
Ser Cys Gly Val Tyr Tyr Glu Cys Ser Glu Thr Pro Val Leu Phe Thr
        675                 680                 685

aag aag aat agg att tat cta tac atc ata ttg gca gta tcg ctt gta      2112
Lys Lys Asn Arg Ile Tyr Leu Tyr Ile Ile Leu Ala Val Ser Leu Val
    690                 695                 700

gta ctg gcc gtc tta gcc tac ttt gga tac agg tac tac agt aag aat      2160
Val Leu Ala Val Leu Ala Tyr Phe Gly Tyr Arg Tyr Tyr Ser Lys Asn
705                 710                 715                 720

cac ttg aaa aaa cac aat tcc cag ata tat gaa gat gat aac gtg aac      2208
His Leu Lys Lys His Asn Ser Gln Ile Tyr Glu Asp Asp Asn Val Asn
                725                 730                 735
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aga tct gaa gtt act cca gtc aga atc tct agg tta aac cat taa 2349
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<212> PRT
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Pro Val Leu Gly Ser Asn Ser Asp Pro Ser Glu Glu Tyr Asp Ser Phe
20 25 30

Gln Gln Asn Val Phe Thr His Gln Pro Thr Gln Leu His Lys Ser His
35 40 45

His Tyr Ile Thr His Gln Lys Lys Thr Ser Gln His Ile Asp Asp Leu
50 55 60

Asn Phe Tyr Asn Gly Lys Phe Asn Gln Lys Ser Arg Ile Gly Pro Gly
65 70 75 80

Lys Val Val Asn Asn Ser Arg Asn Leu Val Glu Gly Glu Thr Leu Ser
85 90 95

Lys Asp Asp Asn Lys Thr Lys Ser Lys Ile Lys Ser Lys Thr Ala Ser
100 105 110

Ile Leu Pro Arg Leu Leu Lys Ser Leu Ser Phe Leu Ala Val Leu Gly
115 120 125

Ser Ile Asn Ser Phe Ser Leu Ala Leu Glu Glu Pro Phe Thr Gln His
130 135 140

Thr Ser Asn Arg Thr Pro Phe Glu Val Ser Leu Ile Gln Ser Asn Ser
145 150 155 160

Ser Leu Ser Pro Ile His Asn Ser Ser Thr Gln Asn Ser Ser His His
165 170 175

Asn Gly Phe Ser Gly Ser Thr Val Asn Asn Thr Ser Leu Ile Glu Thr
180 185 190

Arg Asn Asn Val Leu Asn Arg Thr Leu Gly Arg Phe Gly Ser Phe Leu
195 200 205

Gln Ser Gly Leu Ile Ser Ser Arg Ala Asp Lys Lys Lys Arg Ser Gly
210 215 220

Met Asn Arg Arg Gly Pro Lys Gly Lys Lys Gly Lys Gly Gly Glu Asp
225 230 235 240

Glu Glu Lys Arg Asn Lys Trp Thr Asp Phe Met Ala Lys Phe Asp Ile
245 250 255

Ala Lys Val His Gly Ser Gly Val Tyr Val Asp Leu Gly Glu Ser Ala
260 265 270

Thr Val Gly Ser Tyr Asp Tyr Arg Met Pro Ile Gly Lys Cys Pro Val
275 280 285

Val Gly Lys Ala Ile Ile Leu Glu Asn Gly Ala Asp Phe Leu Ser Ser
290 295 300

Ile Thr His His Asp Pro Lys Glu Arg Gly Leu Gly Phe Pro Ala Thr
305 310 315 320

Lys Val Ala Ser Asn Ser Ser Lys Leu Asp Met Glu Asn Gln Leu Leu
325 330 335

Ser Pro Ile Ser Ala Gln Val Leu Arg Ser Trp Asn Tyr Lys His Glu
340 345 350

Ser Asp Leu Ser Asn Cys Ala Glu Tyr Ser Arg Asn Ile Val Pro Gly
355 360 365

Ser Asn Arg Asn Ser Lys Tyr Arg Tyr Pro Phe Val Tyr Asp Glu Ser
370 375 380

Glu Lys Leu Cys Tyr Ile Leu Tyr Ser Pro Met Gln Tyr Asn Gln Gly
385 390 395 400

[illegible]

Gly Thr Ser Gly Leu His Met Asp Trp Pro Val Val Cys Pro Val Tyr
435 440 445

Pro Ile Arg Asp Ser Ile Phe Gly Ser Tyr Asp Asp Gln Lys Asp Glu
450 455 460

Cys Val Pro Ile Glu Pro Ile Phe Glu Glu Glu Ala Glu Asp Tyr Glu
465 470 475 480

Ala Cys Ala Lys Ile Ile Phe Glu Tyr Ser Pro Ser Asp Val Asp Ile
485 490 495

Ser Thr Asn Asn Gln Lys Leu Ser Asp Val Asp Leu Tyr Lys Glu Ala
500 505 510

Met Asn Asn Gly Lys Leu Ser Thr Ala Leu Ser Ile Met Phe Ala Pro
515 520 525

Arg Tyr Ser Glu Asp Arg Pro Ile Tyr Thr Lys Gly Val Gly Ile Asn
530 535 540

Trp Ala Thr Tyr Ser Val Glu Glu Lys Lys Cys Asn Ile Leu Asp Val
545 550 555 560

Val Pro Ser Cys Leu Ile Ile Ser Asn Gly His Tyr Ala Leu Thr Ser
565 570 575

Leu Ser Ser Pro Asn Glu Glu Asp Ala Ile Asn Tyr Pro Cys Asp Ile
580 585 590

Val Gln Gly Lys Gly Phe Leu Lys Asn Pro Asn Gly Gly Lys Lys Asn
595 600 605

Ala Gln Glu Pro Pro Lys Glu Pro Glu Pro Glu Glu Pro Lys Lys Glu
610 615 620

Gly Ala Glu Asn Lys Pro Lys Glu Lys Gly Lys Ser Glu Lys Lys Asn
625 630 635 640

Glu Lys Ser Met Pro Ser Gly Pro Phe Thr Pro Tyr Thr Ser Leu Lys
645 650 655

Lys Glu Gly Phe Glu Cys Ser Lys Tyr Thr Val Glu Arg Val Asn Lys
660 665 670

Ser Cys Gly Val Tyr Tyr Glu Cys Ser Glu Thr Pro Val Leu Phe Thr
675 680 685

Lys Lys Asn Arg Ile Tyr Leu Tyr Ile Ile Leu Ala Val Ser Leu Val
690 695 700

Val Leu Ala Val Leu Ala Tyr Phe Gly Tyr Arg Tyr Tyr Ser Lys Asn
705 710 715 720

His Leu Lys Lys His Asn Ser Gln Ile Tyr Glu Asp Asp Asn Val Asn
725 730 735

Asn Tyr Tyr Asn Glu Asp Phe Asp Asp Glu Gln Asp Arg Asp Glu Tyr
740 745 750

Ala Ser Asn Val Arg Gly Asp Gln Ile Trp Ser Arg His Thr Pro Asp
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770 775 780

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<213> Babesia bovis

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<222> (1)..(1968)

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gcc ttt tta gct aca acg ggg ata cat gct ttt gcg gac aaa ggt att 96
Ala Phe Leu Ala Thr Thr Gly Ile His Ala Phe Ala Asp Lys Gly Ile
20 25 30

ggt tca cca aag ggg aaa caa tgc aag aag caa ctt gac ttt tcg att 144
Gly Ser Pro Lys Gly Lys Gln Cys Lys Lys Gln Leu Asp Phe Ser Ile
35 40 45

gtg gta gat gaa tct gct agt ata tcg gtt gat caa tgg gag ggt cag 192
Val Val Asp Glu Ser Ala Ser Ile Ser Val Asp Gln Trp Glu Gly Gln

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act gac ata cgt ctt tcg ctt acc act tac tca act cca act cgc cag        288
Thr Asp Ile Arg Leu Ser Leu Thr Thr Tyr Ser Thr Pro Thr Arg Gln
                85                  90                  95

ata ttt acg ttt ttg gat gct gct gca agc agt acc agg ctc gca ctc        336
Ile Phe Thr Phe Leu Asp Ala Ala Ala Ser Ser Thr Arg Leu Ala Leu
            100                 105                 110

acg aaa ctt gat tgg atg aac ggt acc aaa gct agg tat ggt atg acc        384
Thr Lys Leu Asp Trp Met Asn Gly Thr Lys Ala Arg Tyr Gly Met Thr
        115                 120                 125

tac act ggc agg gct ctg aac tac gtt cgt aag gct ata cta cca tat        432
Tyr Thr Gly Arg Ala Leu Asn Tyr Val Arg Lys Ala Ile Leu Pro Tyr
    130                 135                 140

ggt cgc aag aat gta ccc aag gca ctg tta ctg atc act gat gga gta        480
Gly Arg Lys Asn Val Pro Lys Ala Leu Leu Leu Ile Thr Asp Gly Val
145                 150                 155                 160

tct tcg gat gga agt tac act gca cag gtt gcg gct atg ctt cgt gat        528
Ser Ser Asp Gly Ser Tyr Thr Ala Gln Val Ala Ala Met Leu Arg Asp
                165                 170                 175

gaa ggt gta aat gta atg gtt att ggt gtc ggt gat gta aat gtt gct        576
Glu Gly Val Asn Val Met Val Ile Gly Val Gly Asp Val Asn Val Ala
            180                 185                 190

gaa tgc cgt ggc ata gta gga tgt gat gga ata atg gat tgt cct atg        624
Glu Cys Arg Gly Ile Val Gly Cys Asp Gly Ile Met Asp Cys Pro Met
        195                 200                 205

ttc aag cag acc aac tgg aag gat atc atg ggc ctc ttt aac agt tta        672
Phe Lys Gln Thr Asn Trp Lys Asp Ile Met Gly Leu Phe Asn Ser Leu
    210                 215                 220

atg aag gag gta tgt gat att tta cct cag gac gct gtt tgt gag cct        720
Met Lys Glu Val Cys Asp Ile Leu Pro Gln Asp Ala Val Cys Glu Pro
225                 230                 235                 240

gta tgg gca gaa tgg tca tct tgt aac ggg gaa tgt ggc gtt cct ggt        768
Val Trp Ala Glu Trp Ser Ser Cys Asn Gly Glu Cys Gly Val Pro Gly
                245                 250                 255

aaa cga act cgt gct ctt ttg gac ctc cga atg att gaa aag ccc gta        816
Lys Arg Thr Arg Ala Leu Leu Asp Leu Arg Met Ile Glu Lys Pro Val
            260                 265                 270

aat ggc tcg aat gga caa ccg ggt aaa tca tgt gag gat cag aag atg        864
Asn Gly Ser Asn Gly Gln Pro Gly Lys Ser Cys Glu Asp Gln Lys Met
        275                 280                 285

aac ttc tta ccc caa tca gag aca tgc acc ata gaa tgc aat cat gag        912
Asn Phe Leu Pro Gln Ser Glu Thr Cys Thr Ile Glu Cys Asn His Glu
    290                 295                 300

cct gtg cca agc tcg ccg gaa cct gta tca gat gat atg gat cac cca        960
Pro Val Pro Ser Ser Pro Glu Pro Val Ser Asp Asp Met Asp His Pro
305                 310                 315                 320
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gaa cca act cct gtt aca ccg gaa ggt gac atg gat aaa tct cat tcc      1008
Glu Pro Thr Pro Val Thr Pro Glu Gly Asp Met Asp Lys Ser His Ser
                325                 330                 335

cat tcg agc att cca tcc acc cct gat atg cca tca agt cac agt gat      1056
His Ser Ser Ile Pro Ser Thr Pro Asp Met Pro Ser Ser His Ser Asp
            340                 345                 350

atg tca tca agc cct act gat atg tca tca agc cct act gac atg tca      1104
Met Ser Ser Ser Pro Thr Asp Met Ser Ser Ser Pro Thr Asp Met Ser
        355                 360                 365

tca agc cct act gac atg tca tca agt cac agt gac atg cca tca act      1152
Ser Ser Pro Thr Asp Met Ser Ser Ser His Ser Asp Met Pro Ser Thr
    370                 375                 380

cct act ggc atg tca tca agt cac agt gat atg cca tca agt cac agt      1200
Pro Thr Gly Met Ser Ser Ser His Ser Asp Met Pro Ser Ser His Ser
385                 390                 395                 400

gat atg cca tca agc cac agt gat atg tca tca agc cct act gac atg      1248
Asp Met Pro Ser Ser His Ser Asp Met Ser Ser Ser Pro Thr Asp Met
                405                 410                 415

tca tca agt cac gct gat act cgt gta gga aat acc gat gaa gaa cat      1296
Ser Ser Ser His Ala Asp Thr Arg Val Gly Asn Thr Asp Glu Glu His
            420                 425                 430

aac cac agg aaa gat atg gat gtc aag ttc ccc gaa aat atg gat gat      1344
Asn His Arg Lys Asp Met Asp Val Lys Phe Pro Glu Asn Met Asp Asp
        435                 440                 445

atc cca gtc gag gat aat cct ata ccc aca gat cct aga cat ggc gtc      1392
Ile Pro Val Glu Asp Asn Pro Ile Pro Thr Asp Pro Arg His Gly Val
    450                 455                 460

gaa cca tcg cct tct gat gtg atc cct gag gat gac caa ctt cgt agg      1440
Glu Pro Ser Pro Ser Asp Val Ile Pro Glu Asp Asp Gln Leu Arg Arg
465                 470                 475                 480

acg ctt gaa atg cag cgc gaa gag gac cta aag aag gaa ttg atg ctc      1488
Thr Leu Glu Met Gln Arg Glu Glu Asp Leu Lys Lys Glu Leu Met Leu
                485                 490                 495

caa cat gaa ctg aag ctt cag gaa gaa aag gaa agg gca gct att tta      1536
Gln His Glu Leu Lys Leu Gln Glu Glu Lys Glu Arg Ala Ala Ile Leu
            500                 505                 510

gag aat aac act cct tat gga tcc gcc act tcc gtg tcg caa gac ggt      1584
Glu Asn Asn Thr Pro Tyr Gly Ser Ala Thr Ser Val Ser Gln Asp Gly
        515                 520                 525
```

```
aat gct gat gtg acc gaa tct gag gac tat gag ggt gaa aaa caa aag      1728
Asn Ala Asp Val Thr Glu Ser Glu Asp Tyr Glu Gly Glu Lys Gln Lys
            565                 570                 575

gac gaa tca aat gaa cgt tcg acc agc aac act act aag att gcc ggc      1776
Asp Glu Ser Asn Glu Arg Ser Thr Ser Asn Thr Thr Lys Ile Ala Gly
        580                 585                 590

ggt gct cta cta ggt ctt ctt ctc ctt ggt gcc ggt ggt gga tac gct      1824
Gly Ala Leu Leu Gly Leu Leu Leu Leu Gly Ala Gly Gly Gly Tyr Ala
    595                 600                 605

atg tac aaa aag aac aag aca cct act gtt gag aca ggt tca ggt gat      1872
Met Tyr Lys Lys Asn Lys Thr Pro Thr Val Glu Thr Gly Ser Gly Asp
610                 615                 620

tac act ggg gcc gac gag agt tca gaa ccc atg aag gag ggt gac aca      1920
Tyr Thr Gly Ala Asp Glu Ser Ser Glu Pro Met Lys Glu Gly Asp Thr
625                 630                 635                 640

tac acc gtc act gag ttt gac aac aac att tgg ggc gag gca gcg taa      1968
Tyr Thr Val Thr Glu Phe Asp Asn Asn Ile Trp Gly Glu Ala Ala
                645                 650                 655
```

<210> 6
<211> 655
<212> PRT
<213> Babesia bovis

<400> 6

```
Met Ile Gly Tyr Ile Lys Ile Leu Ala Ser Val Pro Leu Leu Ser Leu
1               5                   10                  15

Ala Phe Leu Ala Thr Thr Gly Ile His Ala Phe Ala Asp Lys Gly Ile
            20                  25                  30

Gly Ser Pro Lys Gly Lys Gln Cys Lys Lys Gln Leu Asp Phe Ser Ile
        35                  40                  45

Val Val Asp Glu Ser Ala Ser Ile Ser Asp Asp Gln Trp Glu Gly Gln
    50                  55                  60

Met Ile Pro Phe Leu Arg Asn Leu Ile His Thr Val Asp Leu Asp Asn
65                  70                  75                  80

Thr Asp Ile Arg Leu Ser Leu Thr Thr Tyr Ser Thr Pro Thr Arg Gln
                85                  90                  95

Ile Phe Thr Phe Leu Asp Ala Ala Ala Ser Ser Thr Arg Leu Ala Leu
            100                 105                 110
```

Thr Lys Leu Asp Trp Met Asn Gly Thr Lys Ala Arg Tyr Gly Met Thr
115 120 125

Tyr Thr Gly Arg Ala Leu Asn Tyr Val Arg Lys Ala Ile Leu Pro Tyr
130 135 140

Gly Arg Lys Asn Val Pro Lys Ala Leu Leu Leu Ile Thr Asp Gly Val
145 150 155 160

Ser Ser Asp Gly Ser Tyr Thr Ala Gln Val Ala Ala Met Leu Arg Asp
165 170 175

Glu Gly Val Asn Val Met Val Ile Gly Val Gly Asp Val Asn Val Ala
180 185 190

Glu Cys Arg Gly Ile Val Gly Cys Asp Gly Ile Met Asp Cys Pro Met
195 200 205

Phe Lys Gln Thr Asn Trp Lys Asp Ile Met Gly Leu Phe Asn Ser Leu
210 215 220

Met Lys Glu Val Cys Asp Ile Leu Pro Gln Asp Ala Val Cys Glu Pro
225 230 235 240

Val Trp Ala Glu Trp Ser Ser Cys Asn Gly Glu Cys Gly Val Pro Gly
245 250 255

Lys Arg Thr Arg Ala Leu Leu Asp Leu Arg Met Ile Glu Lys Pro Val
260 265 270

Asn Gly Ser Asn Gly Gln Pro Gly Lys Ser Cys Glu Asp Gln Lys Met
275 280 285

Asn Phe Leu Pro Gln Ser Glu Thr Cys Thr Ile Glu Cys Asn His Glu
290 295 300

Pro Val Pro Ser Ser Pro Glu Pro Val Ser Asp Asp Met Asp His Pro
305 310 315

Met Ser Ser Ser Pro Thr Asp Met Ser Ser Ser Pro Thr Asp Met Ser
355 360 365

Ser Ser Pro Thr Asp Met Ser Ser Ser His Ser Asp Met Pro Ser Thr
370 375 380

Pro Thr Gly Met Ser Ser Ser His Ser Asp Met Pro Ser Ser His Ser
385 390 395 400

Asp Met Pro Ser Ser His Ser Asp Met Ser Ser Ser Pro Thr Asp Met
405 410 415

Ser Ser Ser His Ala Asp Thr Arg Val Gly Asn Thr Asp Glu Glu His
420 425 430

Asn His Arg Lys Asp Met Asp Val Lys Phe Pro Glu Asn Met Asp Asp
435 440 445

Ile Pro Val Glu Asp Asn Pro Ile Pro Thr Asp Pro Arg His Gly Val
450 455 460

Glu Pro Ser Pro Ser Asp Val Ile Pro Glu Asp Asp Gln Leu Arg Arg
465 470 475 480

Thr Leu Glu Met Gln Arg Glu Glu Asp Leu Lys Lys Glu Leu Met Leu
485 490 495

Gln His Glu Leu Lys Leu Gln Glu Glu Lys Glu Arg Ala Ala Ile Leu
500 505 510

Glu Asn Asn Thr Pro Tyr Gly Ser Ala Thr Ser Val Ser Gln Asp Gly
515 520 525

Glu Ser Pro Thr Gly Val Pro Gln Ser Ser Glu Thr Asp Ala Ile Arg
530 535 540

His Glu Val Tyr Asp Asp His Pro Glu Glu Ser Glu Asn Thr Gly Ile
545 550 555 560

Asn Ala Asp Val Thr Glu Ser Glu Asp Tyr Glu Gly Glu Lys Gln Lys
565 570 575

Asp Glu Ser Asn Glu Arg Ser Thr Ser Asn Thr Thr Lys Ile Ala Gly
580 585 590

```
Gly Ala Leu Leu Gly Leu Leu Leu Leu Gly Ala Gly Gly Gly Tyr Ala
        595                 600                 605

Met Tyr Lys Lys Asn Lys Thr Pro Thr Val Glu Thr Gly Ser Gly Asp
    610                 615                 620

Tyr Thr Gly Ala Asp Glu Ser Ser Glu Pro Met Lys Glu Gly Asp Thr
625                 630                 635                 640

Tyr Thr Val Thr Glu Phe Asp Asn Asn Ile Trp Gly Glu Ala Ala
                645                 650                 655
```

<210> 7
<211> 1047
<212> DNA
<213> Theileria annulata

<220>
<221> CDS
<222> (1)..(1047)

<400> 7

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gat aag ggg cta tat cct gac ggt ata aag aaa ccg agc tcc tac tgc      48
Asp Lys Gly Leu Tyr Pro Asp Gly Ile Lys Lys Pro Ser Ser Tyr Cys
1               5                   10                  15

cac agg gaa ttg gac tta aca ata tta gtc gat gaa tcc tcg agt atc      96
His Arg Glu Leu Asp Leu Thr Ile Leu Val Asp Glu Ser Ser Ser Ile
            20                  25                  30

tat att gaa gag tgg aac aaa ctc att cca ttt ctt aaa tca ctg gtg     144
Tyr Ile Glu Glu Trp Asn Lys Leu Ile Pro Phe Leu Lys Ser Leu Val
        35                  40                  45

aga tca ata aat ata agt cca aat tat gtg cac ttg tca atg gtc acc     192
Arg Ser Ile Asn Ile Ser Pro Asn Tyr Val His Leu Ser Met Val Thr
    50                  55                  60

ttt tcc act tca att cgg tgg tta ata tca ttt ctc gac cca gcc tct     240
Phe Ser Thr Ser Ile Arg Trp Leu Ile Ser Phe Leu Asp Pro Ala Ser
65                  70                  75                  80

aag gat gag caa tta gcc ctt gct gtt ctg gac aag ctg aag aac agt     288
Lys Asp Glu Gln Leu Ala Leu Ala Val Leu Asp Lys Leu Lys Asn Ser
                85                  90
```

```
atc att atc atc acc gac gga tcc tct act cag aca aac gtt act tct        432
Ile Ile Ile Ile Thr Asp Gly Ser Ser Thr Gln Thr Asn Val Thr Ser
    130                 135                 140

cag gcg tcg gct cta cta agg gat gct ggt gta aca att cta gtt gtt        480
Gln Ala Ser Ala Leu Leu Arg Asp Ala Gly Val Thr Ile Leu Val Val
145                 150                 155                 160

gga gtt ggg aag gct aaa gaa agc gag tgt aga ggt ata gtt ggt tgt        528
Gly Val Gly Lys Ala Lys Glu Ser Glu Cys Arg Gly Ile Val Gly Cys
                165                 170                 175

tct acc aaa gga gag tgc ccc ctt ttc ttt atg acc aac tgg gat gaa        576
Ser Thr Lys Gly Glu Cys Pro Leu Phe Phe Met Thr Asn Trp Asp Glu
            180                 185                 190

att atc agg aag gtt ggg gag ttg atg gct gag gtt tgt gag acc att        624
Ile Ile Arg Lys Val Gly Glu Leu Met Ala Glu Val Cys Glu Thr Ile
        195                 200                 205

cct aag gac gcc gta tgt aag ccg atc tgg tct gat tgg tct aag tgt        672
Pro Lys Asp Ala Val Cys Lys Pro Ile Trp Ser Asp Trp Ser Lys Cys
    210                 215                 220

gac gcc aag tgc ggc att ggg acg agg tac caa aag ttg atg gga gtt        720
Asp Ala Lys Cys Gly Ile Gly Thr Arg Tyr Gln Lys Leu Met Gly Val
225                 230                 235                 240

act aca att tct gag cca act gtc gga acg aac ggc aag tcc ggg agg        768
Thr Thr Ile Ser Glu Pro Thr Val Gly Thr Asn Gly Lys Ser Gly Arg
                245                 250                 255

aca tgt gag atg att tat gag aac gtc gag gtt cca aag gag gag tgc        816
Thr Cys Glu Met Ile Tyr Glu Asn Val Glu Val Pro Lys Glu Glu Cys
            260                 265                 270

tcc gtt gag tct aag att gct gga gga gtg gct cta gca ctg tta atg        864
Ser Val Glu Ser Lys Ile Ala Gly Gly Val Ala Leu Ala Leu Leu Met
        275                 280                 285

ctt gca ggc gga ggt ggt tac aca tac tac aaa aag tac ggt tta tct        912
Leu Ala Gly Gly Gly Gly Tyr Thr Tyr Tyr Lys Lys Tyr Gly Leu Ser
    290                 295                 300

aga gtg agt gaa act acg aat ttg gat gag gat ttt gca gat tct agt        960
Arg Val Ser Glu Thr Thr Asn Leu Asp Glu Asp Phe Ala Asp Ser Ser
305                 310                 315                 320

ggg aac cgt ggt gta agg gag agt gtg ggt gaa gct tac aca gta act       1008
Gly Asn Arg Gly Val Arg Glu Ser Val Gly Glu Ala Tyr Thr Val Thr
                325                 330                 335

gat tta gat gat gga ctc tgg agc caa tcc aat caa taa                   1047
Asp Leu Asp Asp Gly Leu Trp Ser Gln Ser Asn Gln
            340                 345
```

<210> 8
<211> 348
<212> PRT
<213> Theileria annulata

<400> 8

Asp Lys Gly Leu Tyr Pro Asp Gly Ile Lys Lys Pro Ser Ser Tyr Cys
1 5 10 15

His Arg Glu Leu Asp Leu Thr Ile Leu Val Asp Glu Ser Ser Ser Ile
20 25 30

Tyr Ile Glu Glu Trp Asn Lys Leu Ile Pro Phe Leu Lys Ser Leu Val
35 40 45

Arg Ser Ile Asn Ile Ser Pro Asn Tyr Val His Leu Ser Met Val Thr
50 55 60

Phe Ser Thr Ser Ile Arg Trp Leu Ile Ser Phe Leu Asp Pro Ala Ser
65 70 75 80

Lys Asp Glu Gln Leu Ala Leu Ala Val Leu Asp Lys Leu Lys Asn Ser
85 90 95

Lys Pro Val Phe Gly Tyr Thr Phe Thr Gly Gln Ala Leu Asn Phe Ile
100 105 110

Ser Glu Ala Val Tyr Met Phe Gly Ala Arg Arg Asn Ser Pro Lys Gly
115 120 125

Ile Ile Ile Ile Thr Asp Gly Ser Ser Thr Gln Thr Asn Val Thr Ser
130 135 140

Gln Ala Ser Ala Leu Leu Arg Asp Ala Gly Val Thr Ile Leu Val Val
145 150 155 160

Gly Val Gly Lys Ala Lys Glu Ser Glu Cys Arg Gly Ile Val Gly Cys
165 170 175

[illegible]

Pro Lys Asp Ala Val Cys Lys Pro Ile Trp Ser Asp Trp Ser Lys Cys
210 215 220

Asp Ala Lys Cys Gly Ile Gly Thr Arg Tyr Gln Lys Leu Met Gly Val
225 230 235 240

Thr Thr Ile Ser Glu Pro Thr Val Gly Thr Asn Gly Lys Ser Gly Arg
245 250 255

Thr Cys Glu Met Ile Tyr Glu Asn Val Glu Val Pro Lys Glu Glu Cys
260 265 270

Ser Val Glu Ser Lys Ile Ala Gly Gly Val Ala Leu Ala Leu Leu Met
275 280 285

Leu Ala Gly Gly Gly Gly Tyr Thr Tyr Tyr Lys Lys Tyr Gly Leu Ser
290 295 300

Arg Val Ser Glu Thr Thr Asn Leu Asp Glu Asp Phe Ala Asp Ser Ser
305 310 315 320

Gly Asn Arg Gly Val Arg Glu Ser Val Gly Glu Ala Tyr Thr Val Thr
325 330 335

Asp Leu Asp Asp Gly Leu Trp Ser Gln Ser Asn Gln
340 345

<210> 9
<211> 2259
<212> DNA
<213> Babesia bovis

<220>
<221> CDS
<222> (552)..(2189)

<400> 9
ataagatgta gcactgatgt gtgtactcgg actctgacac tggagtatag gctaccagaa 60

ctgggcgcaa ctccctaatg gagtgccgct cccaggaggc cacagaacaa tggagtacaa 120

cgctcaaaac cgcagtgaat gttagctaca atatgtacat attgtcatgg agttcgtaat 180

cctaacaaag gccattgtat cgtcaatgtg gtctaccagt ggacgtcgct tgtggaggcc 240

agggtacatc aaatccctga gaacacctat cgtccggtgt tacggtggta atgggttact 300

ataaaagcaa atttaattgt agatattgta aaaaaactgt aaaattggtt agtgcttgca 360

ccgtcctggt cccgcgattt ggataccgct gtgctacgct ttgcacggaa tcacgacgtc 420

```
gtgcataacg ctgtgcttat gacttcgtac acatcaaacg actttaactg ccgttggttt      480

atatacgttg gcgttaggtt gttttgggtg ttattgtact gtggaatcat acacattcta      540

cacgtgtcat g atg gtg aag ttc cac aca tta tcg gtt gca gcc atc ctg        590
            Met Val Lys Phe His Thr Leu Ser Val Ala Ala Ile Leu
            1               5                   10

gcg att gct tca tcc aat act att ttt gct aca ttt aga tca aat gga        638
Ala Ile Ala Ser Ser Asn Thr Ile Phe Ala Thr Phe Arg Ser Asn Gly
    15                  20                  25

aaa acc ttc gga gat gaa tct gtt agc ctt cta gaa cat gaa agt acc        686
Lys Thr Phe Gly Asp Glu Ser Val Ser Leu Leu Glu His Glu Ser Thr
30                  35                  40                  45

agt ttg tct cgt ggt cct aga cca acc gaa gat caa atc agt cag tta        734
Ser Leu Ser Arg Gly Pro Arg Pro Thr Glu Asp Gln Ile Ser Gln Leu
                50                  55                  60

cca aaa aat gtt ttc ttt cta ttg gat aac agc att gat atg tct att        782
Pro Lys Asn Val Phe Phe Leu Leu Asp Asn Ser Ile Asp Met Ser Ile
            65                  70                  75

gaa act gga gaa gag aat cgt cat ttc ctc tcc gag ttt ttt aaa ttg        830
Glu Thr Gly Glu Glu Asn Arg His Phe Leu Ser Glu Phe Phe Lys Leu
        80                  85                  90

tta aaa aaa tat gaa gga ata aat gtt tca cta ata agg tac aat agt        878
Leu Lys Lys Tyr Glu Gly Ile Asn Val Ser Leu Ile Arg Tyr Asn Ser
    95                  100                 105

gaa gaa ccg tta ggt tcg acg aaa gca tta acc aac ggg gag ttg aaa        926
Glu Glu Pro Leu Gly Ser Thr Lys Ala Leu Thr Asn Gly Glu Leu Lys
110                 115                 120                 125

aaa cta tcc gat aat att cct act aaa atg cct ttt gac att ggc gtt        974
Lys Leu Ser Asp Asn Ile Pro Thr Lys Met Pro Phe Asp Ile Gly Val
                130                 135                 140

gtt cct act ggt ata gga gct gcc ctc aaa cag ata aaa aca ttg tac       1022
Val Pro Thr Gly Ile Gly Ala Ala Leu Lys Gln Ile Lys Thr Leu Tyr
            145                 150                 155

cct gat cac gaa aag ttc ctt gtt ggg aac acc att act gag ttg gat       1070
Pro Asp His Glu Lys Phe Leu Val Gly Asn Thr Ile Thr Glu Leu Asp
        160                 165                 170

tat tct aaa gca ttg ggt aag gat att gtt gta atc gtg ttt act act       1118
Tyr Ser Lys Ala Leu Gly Lys Asp Ile Val Val Ile Val Phe Thr Thr
    175                 180                 185
```

aaa aac tat tgg act cag cta ttg gga tgc cac tac aat act tgt ttg 1262
Lys Asn Tyr Trp Thr Gln Leu Leu Gly Cys His Tyr Asn Thr Cys Leu
225 230 235

agt tat att cgg gcc aaa ata aca agg cct tca cta tat ctc gat gtt 1310
Ser Tyr Ile Arg Ala Lys Ile Thr Arg Pro Ser Leu Tyr Leu Asp Val
240 245 250

ttg gtg aac agg att gtg tct aaa cgc gcg aaa gat gcc gtt tgt ttg 1358
Leu Val Asn Arg Ile Val Ser Lys Arg Ala Lys Asp Ala Val Cys Leu
255 260 265

gaa gtg tgg acg gat tat aaa cct aac act gaa aaa tcg gat gtg agg 1406
Glu Val Trp Thr Asp Tyr Lys Pro Asn Thr Glu Lys Ser Asp Val Arg
270 275 280 285

att atg act tct acg ttg aaa tta tac aaa acc ctt ctt act gga agc 1454
Ile Met Thr Ser Thr Leu Lys Leu Tyr Lys Thr Leu Leu Thr Gly Ser
290 295 300

ttt gcg gag ara aac atc aaa ggt ctc aca tgt gat gag cag cta aag 1502
Phe Ala Glu Xaa Asn Ile Lys Gly Leu Thr Cys Asp Glu Gln Leu Lys
305 310 315

gat atg cag aaa aga caa ata ttt tgc tac tca aat aag tgt gct ccc 1550
Asp Met Gln Lys Arg Gln Ile Phe Cys Tyr Ser Asn Lys Cys Ala Pro
320 325 330

acg atc tat tca aga tct tat gtt gac tta gct att caa cgt ctt aat 1598
Thr Ile Tyr Ser Arg Ser Tyr Val Asp Leu Ala Ile Gln Arg Leu Asn
335 340 345

gca aaa gat ttt aaa gag gta cta gat gag tca tct tac aga tca cgc 1646
Ala Lys Asp Phe Lys Glu Val Leu Asp Glu Ser Ser Tyr Arg Ser Arg
350 355 360 365

agt ttg caa tca gtg gag aaa cat aat gag caa caa aca ggt tct caa 1694
Ser Leu Gln Ser Val Glu Lys His Asn Glu Gln Gln Thr Gly Ser Gln
370 375 380

gaa acg ctt tct gga agc gcc cgt gta gaa aca agc tta gaa agc tca 1742
Glu Thr Leu Ser Gly Ser Ala Arg Val Glu Thr Ser Leu Glu Ser Ser
385 390 395

gta cct tca tcc tat gtg gca gaa ttg gga gaa agt gat aca gaa aca 1790
Val Pro Ser Ser Tyr Val Ala Glu Leu Gly Glu Ser Asp Thr Glu Thr
400 405 410

tac aaa cag ttg gag tac ata gat aaa aat ggc gtc act gtc ttc aac 1838
Tyr Lys Gln Leu Glu Tyr Ile Asp Lys Asn Gly Val Thr Val Phe Asn
415 420 425

gat gag ccc act gtt gtt gtc gat act ccc gag tac gta caa aag gtg 1886
Asp Glu Pro Thr Val Val Val Asp Thr Pro Glu Tyr Val Gln Lys Val
430 435 440 445

cat gaa aga gaa atg cag ttt gat gaa gaa tcc acc cat ctt ccc aac 1934
His Glu Arg Glu Met Gln Phe Asp Glu Glu Ser Thr His Leu Pro Asn
450 455 460

```
tct ggt aac cac cat cca cct cat cac cga aag ggg gcc aac gga tcc      1982
Ser Gly Asn His His Pro Pro His His Arg Lys Gly Ala Asn Gly Ser
            465                 470                 475

ggt aaa aag acc acg atc gtc gtt ggt att ata tgc ctt gta gta ata      2030
Gly Lys Lys Thr Thr Ile Val Val Gly Ile Ile Cys Leu Val Val Ile
        480                 485                 490

tgc gcc gtc ata gcc ggc gcc tac cta tcc ctt tca cag caa gag tct      2078
Cys Ala Val Ile Ala Gly Ala Tyr Leu Ser Leu Ser Gln Gln Glu Ser
    495                 500                 505

gtg gaa ctc acc tct gaa gag ggt gac ttc ttg aac gac act acg ggt      2126
Val Glu Leu Thr Ser Glu Glu Gly Asp Phe Leu Asn Asp Thr Thr Gly
510                 515                 520                 525

ggt caa cct gag gta ctc gaa aca caa cag gtt gtg gat gca gag aac      2174
Gly Gln Pro Glu Val Leu Glu Thr Gln Gln Val Val Asp Ala Glu Asn
                530                 535                 540

aaa aca tgg ttg taa gacacgaaac gggttgtcac agccaacata tacaaatgca      2229
Lys Thr Trp Leu
            545

gtttaaatta agtcactagt taaaaaaaaa                                    2259
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<210> 10
<211> 545
<212> PRT
<213> Babesia bovis

<220>
<221> misc_feature
<222> (305)..(305)
<223> The 'Xaa' at location 305 stands for Arg, or Lys.

<400> 10

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Met Val Lys Phe His Thr Leu Ser Val Ala Ala Ile Leu Ala Ile Ala
1               5                   10                  15

Ser Ser Asn Thr Ile Phe Ala Thr Phe Arg Ser Asn Gly Lys Thr Phe
            20                  25                  30

Gly Asp Glu Ser Val Ser Leu Leu Glu His Glu Ser Thr Ser Leu Ser
        35                  40                  45
```

Glu Glu Asn Arg His Phe Leu Ser Glu Phe Phe Lys Leu Leu Lys Lys
85 90 95

Tyr Glu Gly Ile Asn Val Ser Leu Ile Arg Tyr Asn Ser Glu Glu Pro
100 105 110

Leu Gly Ser Thr Lys Ala Leu Thr Asn Gly Glu Leu Lys Lys Leu Ser
115 120 125

Asp Asn Ile Pro Thr Lys Met Pro Phe Asp Ile Gly Val Val Pro Thr
130 135 140

Gly Ile Gly Ala Ala Leu Lys Gln Ile Lys Thr Leu Tyr Pro Asp His
145 150 155 160

Glu Lys Phe Leu Val Gly Asn Thr Ile Thr Glu Leu Asp Tyr Ser Lys
165 170 175

Ala Leu Gly Lys Asp Ile Val Val Ile Val Phe Thr Thr Gly His Val
180 185 190

Ile Asp Pro Tyr Leu Ala Tyr Asp Glu Ala Phe Asp Ala Arg Arg Asn
195 200 205

Gly Val Arg Phe Tyr Val Ile Asn Arg Gly Gly Lys Ala Lys Asn Tyr
210 215 220

Trp Thr Gln Leu Leu Gly Cys His Tyr Asn Thr Cys Leu Ser Tyr Ile
225 230 235 240

Arg Ala Lys Ile Thr Arg Pro Ser Leu Tyr Leu Asp Val Leu Val Asn
245 250 255

Arg Ile Val Ser Lys Arg Ala Lys Asp Ala Val Cys Leu Glu Val Trp
260 265 270

Thr Asp Tyr Lys Pro Asn Thr Glu Lys Ser Asp Val Arg Ile Met Thr
275 280 285

Ser Thr Leu Lys Leu Tyr Lys Thr Leu Leu Thr Gly Ser Phe Ala Glu
290 295 300

Xaa Asn Ile Lys Gly Leu Thr Cys Asp Glu Gln Leu Lys Asp Met Gln
305 310 315 320

Lys Arg Gln Ile Phe Cys Tyr Ser Asn Lys Cys Ala Pro Thr Ile Tyr
325 330 335

Ser Arg Ser Tyr Val Asp Leu Ala Ile Gln Arg Leu Asn Ala Lys Asp
340 345 350

Phe Lys Glu Val Leu Asp Glu Ser Ser Tyr Arg Ser Arg Ser Leu Gln
355 360 365

Ser Val Glu Lys His Asn Glu Gln Gln Thr Gly Ser Gln Glu Thr Leu
370 375 380

Ser Gly Ser Ala Arg Val Glu Thr Ser Leu Glu Ser Ser Val Pro Ser
385 390 395 400

Ser Tyr Val Ala Glu Leu Gly Glu Ser Asp Thr Glu Thr Tyr Lys Gln
405 410 415

Leu Glu Tyr Ile Asp Lys Asn Gly Val Thr Val Phe Asn Asp Glu Pro
420 425 430

Thr Val Val Val Asp Thr Pro Glu Tyr Val Gln Lys Val His Glu Arg
435 440 445

Glu Met Gln Phe Asp Glu Glu Ser Thr His Leu Pro Asn Ser Gly Asn
450 455 460

His His Pro Pro His His Arg Lys Gly Ala Asn Gly Ser Gly Lys Lys
465 470 475 480

Thr Thr Ile Val Val Gly Ile Ile Cys Leu Val Val Ile Cys Ala Val
485 490 495

Ile Ala Gly Ala Tyr Leu Ser Leu Ser Gln Gln Glu Ser Val Glu Leu
500 505 510

Thr Ser Glu Glu Gly Asp Phe Leu Asn Asp Thr Thr Gly Gly Gln Pro
515 520 525

Glu Val Leu Ile Thr Gly Glu Val Asp Ala Gln Asn Lys Ile Asp

<210> 11
<211> 22
<212> DNA
<213> Artificial

<220>
<223> primer 1

<400> 11
ccacggctct ggaatctatg tc 22

<210> 12
<211> 24
<212> DNA
<213> Artificial

<220>
<223> primer 2

<400> 12
caaaaggata cctatatttg gtac 24

<210> 13
<211> 27
<212> DNA
<213> Artificial

<220>
<223> Primer 3

<400> 13
tgtggtagat gaatctgcta gtatatc 27

<210> 14
<211> 27
<212> DNA
<213> Artificial

<220>
<223> Primer 4

<400> 14
ctatgccacg gcattcagca acattta 27

<210> 15
<211> 27
<212> DNA
<213> Artificial

<220>
<223> Primer 5

<400> 15
cccggatcca tgcagttaca taacaaa 27

<210> 16
<211> 27
<212> DNA
<213> Artificial

<220>
<223> Primer 6

<400> 16
gggaagcttc tgagcaaagg aaatagg 27

<210> 17
<211> 27
<212> DNA
<213> Artificial

<220>
<223> Primer 7

<400> 17
cccgaattcg tggtagatga atctgct 27

<210> 18
<211> 29
<212> DNA
<213> Artificial

<220>
<223> Primer 8

<400> 18
cccgtcgact gcctcgcccc aaatgttgt 29